Supplemental Methods

Mice

Mfge8$^{-/-}$ (1) and *IL-1β*$^{-/-}$ (2) *Nlrp3*$^{-/-}$ (3) and *P2x7r*$^{-/-}$ (4) mice have been previously described. *β3*$^{-/-}$ mice are from Jackson laboratory (USA). Clotilde Thery (Institut Curie, INSERM U932, Paris, France) provided *Mfge8*$^{-/-}$ mice, Chris Gabel (Amgen, Seattle, USA) and Jean M Kanellopoulos (Université Paris Sud, Orsay, France) provided *P2x7r*$^{-/-}$ mice and Aubry Tardivel (University of Lausanne, Switzerland) provided bone marrow cells from *Nlrp3*$^{-/-}$ mice. All mice were on full C57Bl/6 background. C57Bl/6 mice were used as wild type control. Experiments were performed under French Ministry of Agriculture permit n°02934 and according to the National Institute of Health guidelines for the care of laboratory animals. The study was also approved by the Home Office, PPL 80/2426, United Kingdom.

Chemicals

ATP (A6419), LPS (L4391), Apyrase (A6410), Dexamethasone (D4902) were obtained from Sigma.

Permanent focal cerebral ischemia

Mice were anesthetized by isoflurane. The lateral part of the skull was exposed and a small craniotomy was drilled over the middle cerebral artery (MCA). Left MCA was then electrocoagulated with a bipolar forceps.

Intra-cerebro-ventricular (i.c.v.) injections

A craniotomy was made 0.9 mm right lateral and 0.1 mm posterior relative to the bregma. Injections were performed 2 mm deep. Beginning at D0, we injected on a daily basis 2 µl of either control vehicle (artificial cerebrospinal fluid, aCSF) or 1 *µ*g of rMfge8 protein (R&D Systems). For IL-1ra experiments, 2 *µ*l of aCSF or 5 *µ*g of IL-1ra (AbCys) diluted in aCSF were injected immediately after MCA electrocoagulation.

Assessment of infarct volume

At D7 after MCA electrocoagulation, mice were perfused transcardially with paraformaldehyde. After cryoprotection, 30 mm thick coronal sections were cut. Every 8th section was stained with Cresyl violet. Infarct volume was determined by integrating measured areas and distances between sections, using NIH Image J analysis software. Measured infarct volume was corrected for edema with the following formula: infarct volume = measured infarct volume * (controlateral hemisphere volume/ipsilateral hemisphere volume).

In vitro ATP stimulation and treatments

Wild type bone marrow cells were obtained from 6-week old male C57Bl/6 mice. Bone marrow differentiated macrophages were differentiated as previously described (5). The BMDM was confirmed by flow cytometry with CD11c (BD Pharmingen) and F4/80 (eBiosciences). 2 x 10^6 differentiated BMDM cells from either WT, $\beta 3^{-/-}$ or *P2x7r*$^{-/-}$ mice were primed for 6 hours in the presence of 100 ng/mL LPS unless otherwise specified. Cells were then incubated with indicated amount of Mfge8 (R&D Systems) prior to ATP

stimulation. Endotoxin levels of the recombinant Mfge8 were below 1.0 EU per 1μg of protein. ATP stimulation (5mM) was performed for 30 minutes. Cell media were collected for ELISA assay. Cells were scraped and collected for Caspase 1 colorimetric assay.

In vitro necrotic cell co-culture and Apyrase treatment

Thymus was collected from 6-week old male C57Bl/6 mice. Thymocyte single cell suspension was performed and apoptosis was induced by 10 uM Dexamethasone for 6h as previously described (6). Apoptosis/necrosis was analyzed by using Annexin-V apoptosis detection kit FITC (eBioscience), following manufacturer's instruction. At the stage thymocyte collection after dexamethasone treatment, 30 % of cells were Annexin-V^+ and PI^+, and 31% of cells were Annexin-V^+ and PI^-. Differentiated BMDM were primed with 100 ng/mL LPS for 6 hours. Prior to the co-culture, the BMDM were treated with Apyrase (40 units/mL). Late apoptotic/necrotic (NC) thymocytes were then co-cultured with BMDM (NC:Macrophage = 30:1) for 18 hours. The cell media was collected and IL-1β ELISA was performed.

Western blotting and ELISAs

At D3 after MCA electrocoagulation, left hemispheres were homogenized in a 1% triton buffer for Caspase-1 colorimetric assay (Tris-HCl pH 8.0 50mM, NaCl 150mM, Triton X-100 1%, EDTA 5mM, anti-protease and anti-phosphatase) or a RIPA-SDS buffer for western blotting (Tris-HCl pH 8.0 50 mM, NaCl 150 mM, Triton X-100 1%, SDS 0.2%, anti-protease and anti-

phosphatase, sodium deoxycholate 0.5%). Western blotting was performed with goat anti-IL-1β antibody (R&D, USA). Results are given as a ratio between IL-1β and GAPDH chemiluminescence. Caspase-1 activity was assayed by use of Caspase-1 colorimetric assay kit (Abcam). Mouse IL-1β ELISA set was obtained from BD biosciences (BD OptEIA^{TM}). ELISA was performed according to manufacturer's instruction and analyzed using PerkinElmer victor multilabel plate reader.

Quantitative IL-1β bead assay

Measurement of brain IL-1β in *Mfge8*$^{-/-}$ mice was performed at D3 after MCA electrocoagulation. Left hemispheres were homogenized in a TNE lysis buffer (Tris-HCl pH 8 10mM, NaCl 150mM, EDTA 1mM, Igepal 1%, anti-protease and anti-phosphatase). IL-1β quantitative assay was performed using a Flowcytomix kit (eBioscience, USA) and a Fortessa cell analyser (BD, USA) using manufacturer's instruction. Results are given as pg of IL-1β/mg of total protein.

Real-time PCR analysis

Mice were sacrificed at D3 and RNAs were isolated from the left hemispheres with RNEasy (Qiagen). Ready-to-use primers for IL-1β, TNFα, IL-10, TGFβ and cyclophilin A were used (Super Array, Qiagen). We normalized the results to the level of the housekeeping gene encoding cyclophilin A.

Proliferation assessment

Mice were given 100mg/kg dose of BrdU (Sigma) at 4 h and 2 h before sacrifice.

Immunohistochemistry

Immunohistochemical analyses were performed at D7 after MCA electrocoagulation. Primary antibodies included: anti-BrdU (AbCys), anti-Iba1 (Wako Pure Chemical Industries), anti-CD68 (AbDSerotec), anti-CD31 (BD Biosciences) and anti-myeloperoxidase (MPO) (Abcam). Apoptosis assay was performed using Roche In Situ Cell Death Detection Kit (Roche).

BMDM cells were grown on the 8–well millicell EZ slide (Millipore). After treatment, cells were fixed with 4% paraformaldehyde. Cells were applied with primary P2X7R antibody (Hano43) (NBP1-40894, NOVUS Biologicals) and integrin β3 antibody (ab75872, abcam) overnight at 4°C. Fluorescently-conjugated secondary antibodies were incubated for 1 hour at room temperature. Confocal image was taken using Carl Zeiss LSM 700. Overlap coefficient and signal intensity were analyzed by using Zen software. Overlap coefficient quantifies co-localization in image pairs, and this parameter is insensitive to differences in signal intensities.

Duolink and image analysis

In situ proximity ligation assay was performed according to manufacturer's instruction (Olink). Bone marrow derived macrophages were washed, fixed with 4% paraformaldehyde. Macrophages were then incubated with a pair of primary antibodies overnight at 4°C. Antibodies used are P2RX7 antibody,

integrin beta3 antibody. CD36 antibody (ab78054, abcam) was used as control for the specificity of association between P2RX7 and integrin beta3. Cells were then washed and labeled with (+) strain oligonucleotide-conjugated secondary antibodies and (-) strain oligonucleotide-conjugated secondary antibodies. After addition of template oligonucleotide, annealing, and ligation, the circularized template was amplified via polymerase, and the amplified sequence detected by hybridization with a Green probe. Cells were mounted with Duolink mounting medium. The cells were examined by Carl Zeiss LSM 700 confocal microscope. The threshold was set up in ImageJ software, and the number of spots per cell was counted for analysis.

Efferocytosis assessment

Association between $CD68^+$ cells (macrophage/microglia) and $TUNEL^+$ cells was determined on three 40X magnification fields by an investigator blinded to sample identity. Results are given as the mean percentage of $TUNEL^+$ cells associated with $CD68^+$ cells per region of interest (ROI). To assess internalization, 100 x 0.3 μm consecutive thick deconvoluted pictures were taken for each ROI using a Zeiss Apotom (Imager Z1 with Apotom, Carl Zeiss International) and Axiovision (v4.6.3.0, Carl Zeiss International). The extracellular (i.e. not internalized) or intracellular (i.e. internalized) location of each $TUNEL^+$ nucleus in relationship with the $CD68^+$ cell, was then determined by an investigator blinded to sample identity. $TUNEL^+$ cells only partially included on the z plan were excluded from analysis. Results are given

as the mean percentage of intracellular cells among the $TUNEL^+$ cells associated with $CD68^+$ cells.

Statistics

Statitstical analyses were performed with Prism 5 software (GraphPad). All data are expressed as mean ± SD. Comparisons of two different groups were analyzed by Mann-Whitney U test. For more than two groups, we used ANOVA test with Bonferroni's post-test analysis. A $P < 0.05$ was considered statistically significant.

References

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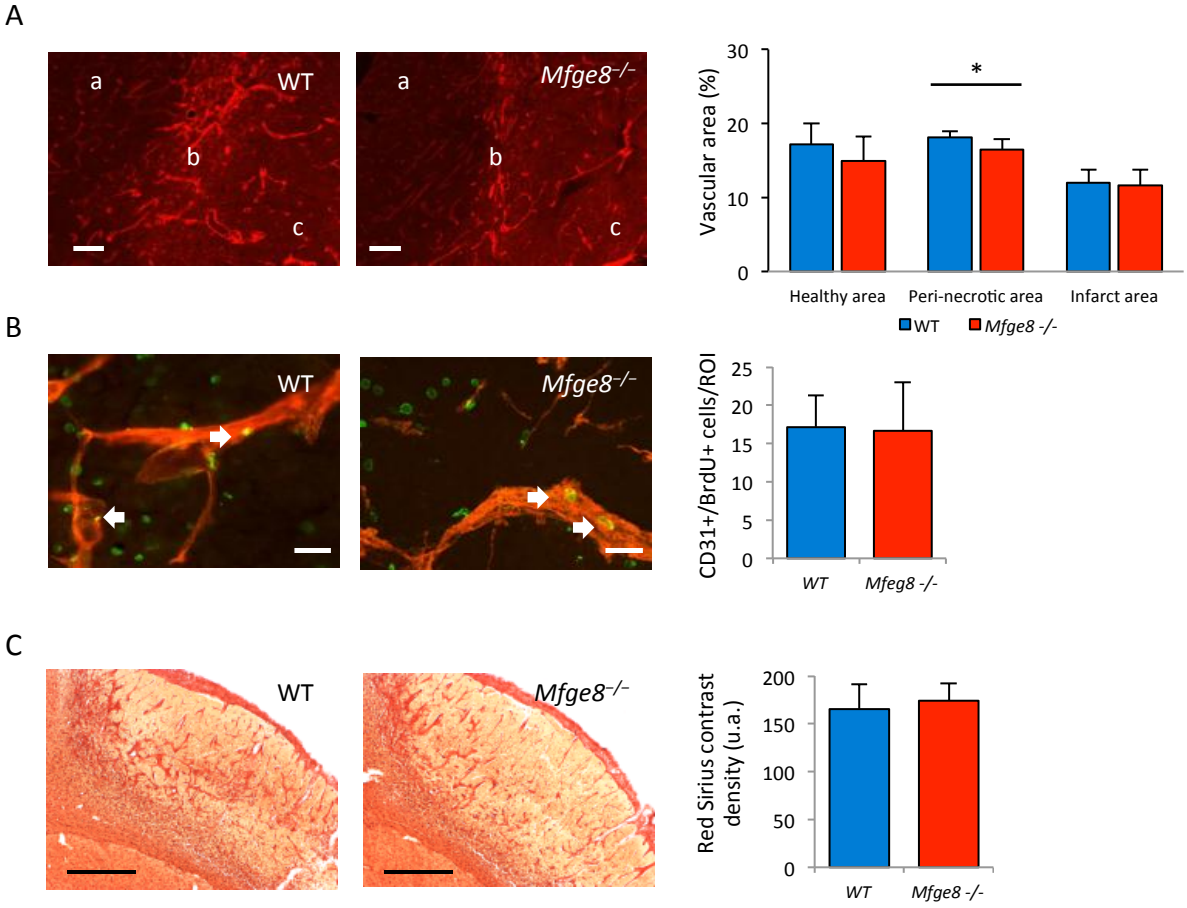
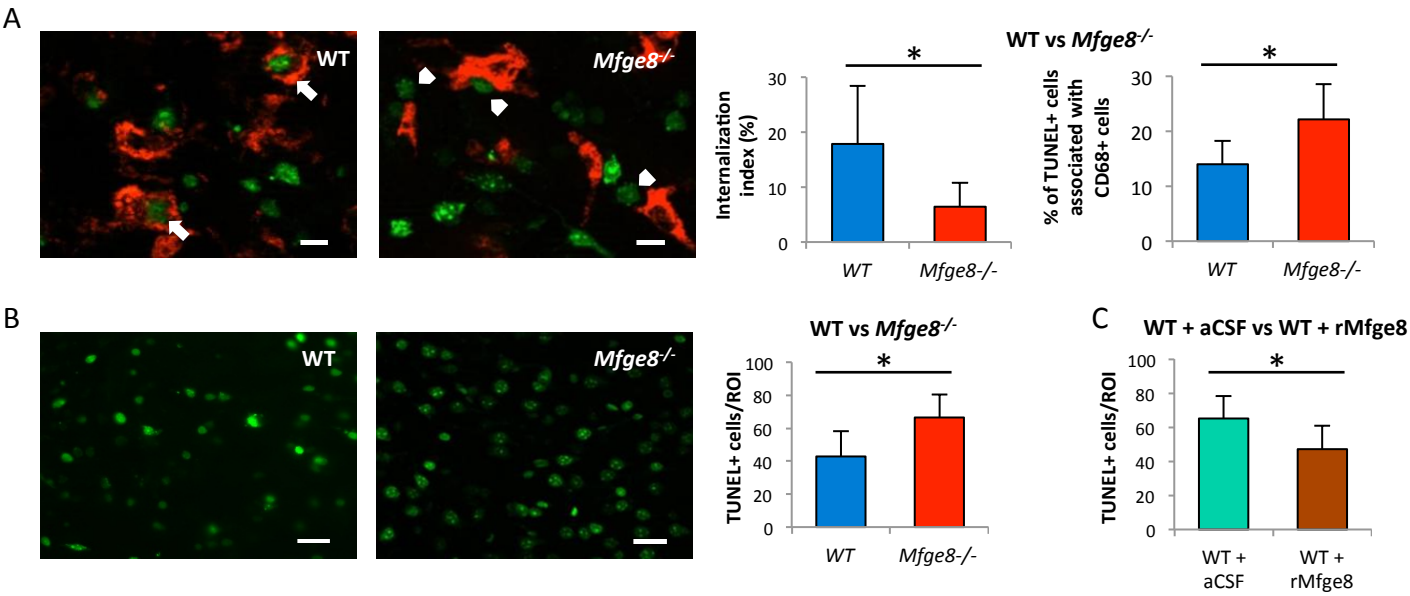
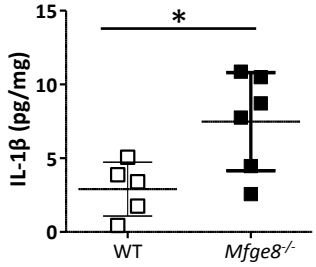


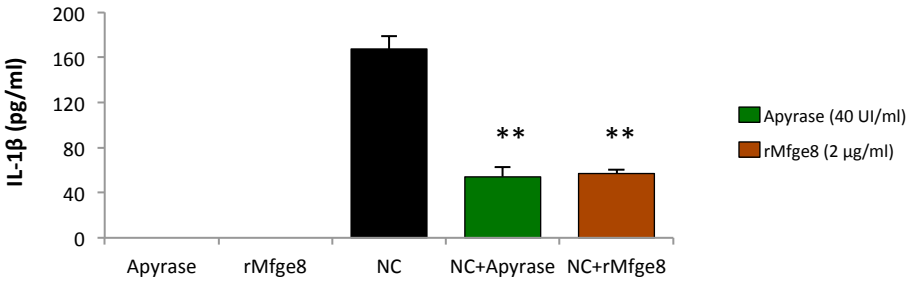
Supplemental Figure 1. **Mfge8 does not significantly alter post-ischemic neovascularization and fibrotic tissue response after cerebral ischemia**. (**A**) Representative photomicrographs and quantification of capillary density after CD31 staining in 3 different areas: a) healthy cerebral parenchyma, b) peri-necrotic area and c) infarct area. Scale bar: 100 µm. (**B**) Double staining for BrdU+ (green) and CD31+ (red) cells. Scale bar: 50 µm. (**C**) Representative photomicrographs and quantification of Red Sirius staining in the 2 groups of mice. Scale bar: 500 µm. * $P < 0.05$; n = 7 to 8 mice per group.



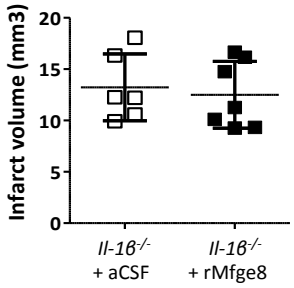
Supplemental Figure 2. Effect of Mfge8 on efferocytosis. (**A**) Efferocytosis was assessed with CD68 (red) and TUNEL (green) double-labeling in the peri-infarct area at D7. The number of fully internalized dead cells (see *Methods*) was significantly reduced in *Mfge8*$^{-/-}$ mice, indicating impaired efferocytosis. This was mirrored by significant accumulation of non-internalized TUNEL+ cells associated to CD68+ cells in *Mfge8*$^{-/-}$ mice. Arrows indicate fully internalized dead cells ; arrowheads indicate associated but not internalized dead cells. Scale bar: 25 µm. (**B, C**) Accumulation of dead cells was quantified in the infarct area using TUNEL staining in the different experimental groups. Scale bar: 50 µm. * $P < 0.05$; n = 7 to 8 mice per group.



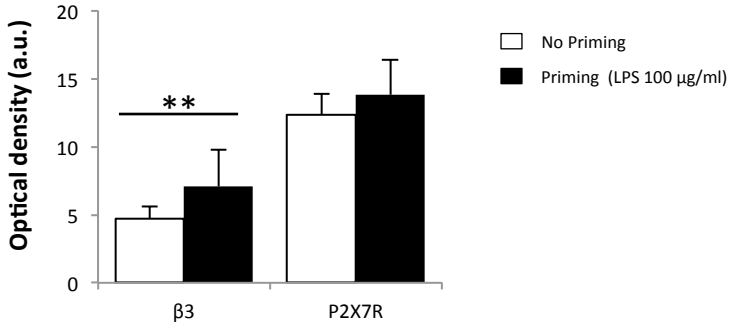
Supplemental Figure 3. IL-1β production in ischemic brains. Brain IL-1β protein level is significantly increased in *Mfge8*$^{-/-}$ mice at D3 after artery occlusion (pg/mg of total protein as measured with Flowcytomix (see Methods).



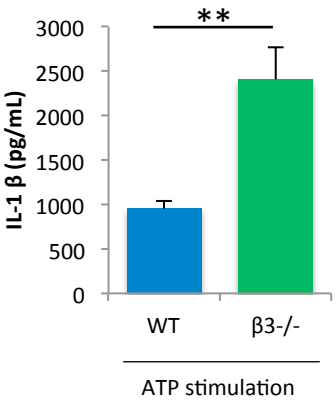
Supplemental Figure 4. rMFG-E8 inhibits necrotic cells-induced IL-1β production in BMDM. BMDM are primed with LPS (see Methods). Necrotic cells (NC) induce apyrase-sensitive IL-1β production by BMDM, indicating an ATP-dependent process. rMfge8 (2 µg/ml) inhibits IL-1β production by BMDM. **: $P < 0.01$; data are representative of 3 independent experiments.



Supplemental Figure 5. Supplementation with recombinant Mfge8 does not reduce infarct size in *Il-1β*-deficient mice. Quantification of infarct volume in *Il-1β*$^{-/-}$ mice treated or not with rMfge8 (administered in artificial cerebrospinal, aCSF, used as vehicle). rMfge8 has no protective effect in the absence of IL-1β.



Supplemental Figure 6. Assessment of β3 and P2X7 expression. Semi-quantitative evaluation of immunofluorescence staining for β3 and P2X7 in BMDM before and after LPS priming. We observed a modest increase of β3 expression after priming. **: $P < 0.01$.



Supplemental Figure 7. β3 expression modulates IL-1β production in BMDM after ATP stimulation. BMDM were obtained from WT or β3-deficient mice, were primed with LPS (see Methods) then stimulated with ATP. **: $P < 0.01$; data are representative of 2 independent experiments.