Supplementary Information

Tissue-plasminogen activator attenuates Alzheimer's disease-related pathology development in APPswe/PS1 mice

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Supplementary Material and Methods

Animal experiments

Experiments were performed according to the Canadian Council on Animal Care guidelines, as administered by the Laval University Animal Welfare Committee. All efforts were made to reduce the number of animals used and to avoid their suffering. Four month old APPswe/PS1 transgenic mice harboring the human presenilin I (A246E variant) and the chimeric mouse/human Aβ precursor protein (APP695swe) under the control of independent mouse prion protein (PrP) promoter elements [B6C3-Tg(APP695)3Dbo Tg(PSEN1)5Dbo/J] (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) maintained in a C57BL/6J background. C57BL6/J mice (wildtype) littermates were used as controls. Additional set of green fluorescent protein $(GFP)^{+/-}$ mice were used to generate chimeric mice. Mice were housed and acclimated to standard laboratory conditions (12-hour light/dark cycle / lights on at 7:00 AM and off at 7:00 PM) with free access to chow and water. Only males were used at the age of 4 months. Four months old mice were used as around this stage the plaques begin to develop in the mouse line used in the laboratory. APPswe/PS1 mice were treated intravenously with a single dose of 5 mg/kg per week of Activase® rt-PA (Roche, Mississauga, ON, Canada) over a total period of ten weeks. As rt-PA has a short half-life in

circulation and could trigger BBB breakdown following administration in stroke, a regimen of chronic low doses that represent half the dose usually used for ischemic stroke thrombolysis, has been chosen. Twenty-four hours after last rt-PA injection, blood was collected from awaken mice via the facial vein for further analysis. Additional group of APPswe/PS1 mice were used to collect blood 3 hours following rt-PA injection for further analysis. The week that followed blood collection, behavioral analysis was performed, mice were then killed and tissues were collected as described here below. In another set of experiments, wildtype mice were treated intravenously with a single 5 mg/kg dose of Activase® rt-PA, 3 and 24 hours after injection blood was collected from awaken mice via the facial vein for further analysis, mice were then killed and tissues were collected as described here below. Brain tissue samples of the mice that were killed 3 hours after injection, were used to assess the level of human rt-PA. A group of APPswe/PS1 chimeric mice, in which the original bone marrow-derived cells were replaced by bone marrow-derived cells of $GFP^{+/-}$ mice, were generated by a myeloablative chemotherapy regimen that preserves the BBB, which were treated with Activase® rt-PA and killed 24 hours following injection. Another group of APPswe/PS1 chimeric mice were generated by total-body irradiation that alters the BBB, which were used as positive controls for GFP-positive blood-borne cell infiltration into the brain. Brain tissues were collected as described here below. Additionally, two groups of APPswe/PS1 mice were treated with a single 5mg/kg dose of Activase® rt-PA, 3 and 24 hours following injection, mice were killed and brains removed and directly processed to isolate microglia for flow cytometry analysis. Finally, a group of wildtype mice were treated with a high dose of rt-PA (10 mg/kg), 3 hours following injection, mice were killed and brains removed and directly processed to assess the level of human rt-PA. The brain tissue samples of these mice were used as a positive control in assessing the entry of systemically administered rt-PA into the brain.

Generation of chimeric mice

APPswe/PS1 chimeric mice were generated by transplanting bone marrow-derived cells of $GFP^{+/-}$ mice in myeloablated APPswe/PS mice, as described previously (Lampron et al., 2013). Briefly, APPswe/PS1 recipient mice were given water containing a commercially available mix of antibiotics (SEPTRA; GlaxoSmithKline, Mississauga, Ontario, Canada) for 1 week before starting a myeloablative chemotherapy regimen consisting of twice-daily injections (morning and evening) of 10 mg/kg busulfan for 4 days (a total of 80 mg/kg), followed by daily injections of 100 mg/kg cyclophosphamide for 2 days (a total of 200 mg/kg). The injections were performed in a total volume of 150 µl intraperitoneally, alternating sides between each injection. To counter chemotherapy-induced dehydration, mice received a daily 1 mL injection of saline subcutaneously for 1 week. Mice were then transferred to sterile cages and given previously irradiated food. Antibiotic treatment continued for 1 week following treatment. On the day following the last injection of cyclophosphamide, $GFP^{+/-}$ donor mice were killed by cervical dislocation with isoflurane anesthesia. Their femurs and tibias were dissected, and their bone marrow was flushed with phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). The cells were filtered through 35-µm nylon mesh (BD Bioscience, San Jose, CA, USA), washed three times in FBS-free PBS (centrifuging at 300*g* for 5 minutes between washes), and counted with a hematocytometer. Cells (1.5×10^7) were then injected into the tail vein of recipient mice. They were followed for 4-6 weeks before they received any other treatment or surgeries to allow the injected cells to repopulate the hematopoietic system. Another group of APPswe/PS1 chimeric mice were generated by exposition to a 10 gray total-body irradiation using a cobalt-60 source (Theratron-780 model, MDS Nordion, Ottawa, ON, Canada), instead of chemotherapy.

Tissue collection

For molecular analysis, mice were deeply anesthetized via an intraperitoneal injection of a mixture of ketamine hydrochloride/xylazine (100/10 mg/kg) and then transcardially perfused with ice-cold 0.9% saline solution (0.9% NaCl) (Sigma-Aldrich) by using a peristaltic pump, brains were removed and immediately processed for microglia isolation or frozen in dry ice for subsequent molecular analysis. For immunofluorescence and histochemical analysis, mice were anesthetized as above and then transcardially perfused with ice-cold 0.9% saline solution, followed by 4% paraformaldehyde (PFA, Sigma-Aldrich) in 0.1 M PBS, brains were removed and postfixed in 4% PFA (pH 7.4) at 4 °C and then immersed in a PFA solution containing 20% sucrose overnight at 4 °C. Fixed brains were frozen with dry ice/ethanol mixture, mounted on a microtome (Leica, Concord, ON, Canada) and cut into 25 µm coronal sections. The collected slices were placed in tissue cryoprotectant solution containing 0.05 M sodium phosphate buffer (pH 7.3), 30% ethylene glycol, and 20% glycerol, and stored at −20 °C until analysis. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) coated tubes during the protocol via the facial veins, and mice were allowed to rest one week after bleeding.

Immunofluorescence staining

Free-floating sections were washed with potassium phosphate-buffered saline (KPBS) (Sigma-Aldrich) (3x, 10 minutes) and then incubated for 20 minutes in a permeabilization/blocking solution containing 4% goat serum, 1% bovine serum albumin (BSA) (Sigma-Aldrich), and 0.4% Triton X-100 (Sigma-Aldrich) in KPBS. Sections were incubated overnight at 4 °C with different primary antibodies diluted in the same permeabilization/blocking solution. The following primary antibodies were used; mouse anti-human Aβ monoclonal antibody (6E10) (1/1500) (SIG-39320, Covance Inc., Princeton, NJ,

USA), rabbit anti-Iba1 (1/1500) (019-19741; Wako Chemicals, Richmond, VA, USA), rabbit anti-CD45 antibody (BD Bioscience). Afterwards, the sections were rinsed in KPBS (3x, 10 minutes), followed by a 2 hours incubation with either Cy3-conjugated goat anti-mouse secondary antibody (115-165-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), or Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (A11008; Life Technologies Inc., Burlington, ON, Canada). Sections were incubated overnight under light protected vacuum to allow an optimal fixation of brain sections on slides. The next day, sections were rinsed in KPBS (3x, 10 minutes), stained with 0.0002% DAPI for 5 minutes, rinsed again in KPBS (3x, 10 minutes), mounted onto SuperFrost slides (Fisher Scientific, Ottawa, ON, Canada), and coverslipped with antifade medium composed of 96 mM Tris-HCl, pH 8.0, 24% glycerol, 9.6% polyvinyl alcohol, and 2.5% diazabicyclooctane (Sigma-Aldrich). Epifluorescence images were taken using a Nikon C80i microscope equipped with both a motorized stage (Ludl, Hawthorne, NY, USA) and a Microfire CCD color camera (Optronics, Goleta, CA, USA). Confocal laser scanning microscopy was performed with a BX-61 microscope equipped with the Fluoview SV500 imaging software 4.3 (Olympus America Inc., Melville, NY, USA).

IgG and albumin extravasation

Free-floating sections were washed with KPBS (3x, 10 minutes) and then incubated for 20 minutes in the permeabilization/blocking solution containing 4% goat serum, 1% bovine serum albumin (BSA) (Sigma-Aldrich), and 0.4% Triton X-100 (Sigma-Aldrich) in KPBS. For IgG detection, sections were incubated for 2 hours with biotin-conjugated goat anti-mouse secondary antibody (1/1000) (BA9200; Vector Laboratories, Burlingame, CA, USA). For albumin detection, sections were incubated overnight at 4 °C with anti-mouse serum albumin (1/1000) (ab19194; Abcam Inc., Toronto, ON, Canada) diluted in the same

permeabilization/blocking solution. The Biotin-conjugated secondary antibodies were detected using the avidin peroxidase kit (Vectastain ABC kit, Vector Laboratories) and diaminobenzidine (Sigma-Aldrich), following the manufacturer's instructions. Sections were then mounted onto SuperFrost slides (Fisher Scientific), dehydrated and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). Bright light images were taken using the Nikon C80i microscope equipped with the motorized stage (Ludl) and a Microfire CCD color camera (Optronics).

Aβ plaques, microglia coverage and Aβ internalization by microglia quantification

Aβ plaques were stained with an anti-human Aβ monoclonal antibody (6E10) as described previously (Simard et al., 2006). Aβ plaque number and size were assessed in the hippocampus and the overlaying cortex separately using a stereological apparatus as described (Boissonneault et al., 2009). Briefly, real-time images (1600 × 1200 pixels) were obtained using the Nikon C80i microscope equipped with the motorized stage (Ludl) and a Microfire CCD color camera (Optronics). Both cortex and hippocampus areas were traced using a Cintiq 18S interactive pen display (Wacom, Ontone, Saitama, Japan). The contours of the cortex or hippocampus areas were traced as virtual overlay on the steamed images. Aβ plaque number and area occupied by Aβ immunostained plaques within these traced virtual regions were determined. In order to assess microglia coverage of Aβ plaques, immunostained brain sections (sections through the hippocampus region) were analyzed using the stereological apparatus as described previously (Boissonneault et al., 2009). Briefly, four brain sections per animal stained for microglia (Iba1), Aβ (6E10) and nuclei (DAPI) were blindly assessed. Aβ plaques were traced and microglia counted for each frame using the pen display, and a 40× Plan Apochromat objective (NA 0.95). In order to investigate Aβ internalization by microglia *in vivo* following rt-PA chronic treatment, Aβ-immunoreactive

microglia were assessed by quantifying Aβ positive immunosignals in microglial cell body. These methods generate semi-quantitative data that are highly representative of the general state of the animal (Simard et al., 2006). Moreover, this method was designed to ensure that this type of quantification was representative of the total amount of plaques and microglia determined by unbiased stereological quantification (Simard et al., 2006). Additional brain sections were double stained for CD45 (infiltrating leukocytes) and Iba1 (differentiated microglia) in order to investigate the infiltration of blood-derived monocytes into the brain and their differentiation into macrophages.

In situ Hybridization

Brain sections were mounted on Colorfrost/Plus microscope slides (Fisher Scientific). *In situ* hybridization histochemical localization of inhibitor of kappa B alpha (IκBα), which is used as an index of nuclear factor-kappa B (NF-kB) activity, was performed using ^{35}S-labeled cRNA probes. Plasmids were linearized and sense/ antisense cRNA probes were synthesized with an appropriate RNA polymerase. All plasmids were analyzed for sequence confirmation and orientation. Riboprobe synthesis and preparation as well as *in situ* hybridization were performed according to a protocol described previously (Laflamme et al., 1999). All slides were developed on the same films that were scanned and densitometrically analyzed using ImageJ image analysis software (NIH).

Human t-PA, and Soluble $A\beta_{1-42}$ / $A\beta_{1-40}$ Enzyme-Linked Immunosorbent Assays (ELISA)s

Brain levels of rt-PA were assessed by using the specific human t-PA Platinum ELISA kit (eBioscience inc., San Diego, CA, USA). Brain levels of soluble $A\beta_{1-42}$ and $A\beta_{1-40}$ were quantified by using the Human Amyloid β42 and Human Amyloid β40 Brain ELISA kits

(Millipore, Billerica, MA, USA). The experimental procedure for human t-PA, $A\beta_{1-42}$ and $A\beta_{1-40}$ detection was performed according to the manufacturer's instructions. Briefly, brains were homogenized in ice cold lysis buffer, and centrifuged at 2500x*g* for 10 minutes at 4 °C. Supernatant was diluted and loaded into 96 wells microplate. Absorbance was acquired using a microtiter plate reader using a 450 nm wavelength filter (SpectraMax 340PC, Molecular Devices, San Diego, CA, USA), and analyzed using SOFTmax Pro3.1.1 software (Molecular Devices).

Brain microvessel isolation

Brain capillaries were isolated on dextran gradient as described previously (ElAli and Hermann, 2010), with slight modification. Briefly, the cerebellum, meninges, brainstem and large superficial blood vessels were removed and the remaining cortices were gently homogenized in a Teflon glass homogenizer in ice-cold microvessel (i.e. capillaries) isolation buffer (MIB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM CaCl2, and 12 mM MgCl2) supplemented with 5% Protease Inhibitor Cocktail (P8340; Sigma-Aldrich) and 1% Phosphatase Inhibitor Cocktail 2 (P5726; Sigma-Aldrich). Homogenates were centrifuged at 1000x*g* for 10 min at 4°C. The resulting pellets were resuspended in 30% dextran (molecular weight, 64,000 to 76,000; D4751, Sigma) in MIB. Suspensions were centrifuged at 4400x*g* for 20 min at 4°C. The resulting crude brain capillaries-rich pellets were resuspended in MIB and filtered through two nylon filters of 100- and 60-µm mesh size (Millipore). The quality of trapped brain in the final filtrates was checked with a bright-field microscopy. Filtrates were centrifuged at 1000x*g*, and the resulting pellets that contain the pure brain capillaries were stored at −80°C until further use.

Isolation and analysis of microglia by flow cytometry

Mice were killed as described previously. Brains were removed and homogenized in 1 mL ice cold Dulbecco's PBS (DPBS) without calcium (Ca^{2+})/ magnesium (Mg^{2+}). Homogenized brain samples were washed for 10 minutes at 300x*g* at 4°C, and resuspended in 3 mL digestion buffer containing a cocktail of enzymes, TLCK (0,5 µg/mL) (Sigma), DNAse1 (25 ng/mL) (Roche), Liberase (8,125pg/mL) (Roche), HEPES 20mM (Sigma) in 0.1 M DPBS, and incubated for 40 minutes at 37°C. Afterwards, the volume was completed to 10 mL by adding 7 mL of flow cytometry buffer containing 5% FBS, EDTA (20 mM), HEPES (2 mM) in flow cytometry buffer. The digested samples were filtered through a 70 µm sterile nylon filter, washed once with flow cytometry buffer, and centrifuged for 10 minutes at 300x*g* at 4°C. The resulting pellet was resuspended in 8 mL 30% Percoll (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada), which was diluted in a solution containing EDTA (2 mM), HEPES (20 mM), phenol red, 1X Hank's balanced salt solution **(**HBSS). 4 mL of the 30% Percoll containing samples were loading carefully at the bottom of a 15 mL falcon tube containing 4 mL 80% Percoll solution, which was diluted in a solution containing EDTA (2 mM), HEPES (20 mM) (Sigma) and 1X HBSS. The remaining 4 mL of the 30% Percoll containing samples were carefully added at the surface of the 80% Percoll solution. Afterwards, the gradient column was centrifuged for 40 minutes at 1000x*g* at 18°C with low acceleration and no brake. Microglial cell enriched fractions were collected from the inter-phase, resuspended in 10 mL of flow cytometry buffer, and centrifuged for 10 minutes at 300x*g*. The resulting pellet, which contains the pure fraction of microglia, was resuspended in 250 µL of flow cytometry buffer, and transferred to a 96-wells conical plate. Cells were incubated for 15 min on ice with rat anti-mouse CD16/CD32 antibody (Mouse BD Fc Block; BD Bioscience) diluted in flow cytometry buffer. Afterwards, cells were centrifuged for 3 min at 300x*g* at 4°C, and incubated for 30 minutes with PE (phycoeritrin)-Cy5-conjugated anti-CD45 antibody (BD Bioscience),

Alexa Fluor® 700-conjugated anti-CD11b antibody (eBioscience, San Diego, CA, USA), and LIVE/DEAD® blue fluorescent dye (Life technologies). Cells were incubated with the antibody mixture for 30 minutes at 4°C. Cells were washed for 10 minutes at 300x*g* with DPBS without Ca^{2+}/Mg^{2+}, and resuspended in flow cytometry buffer. Finally, the cells were analyzed using a LSR II flow cytometer and data acquisition was done with BD FACS Diva software (Version 6.1.2, BD Bioscience). The results were analyzed using FlowJo software (Version 7.6.1, Tree Star Inc., Ashland, OR, USA).

Protein extraction

Isolated brain capillaries or brain tissues were homogenized and lysed in NP-40 lysis buffer supplemented with 5% Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). Lysate samples were sonicated over two cycles lasting 20 s each at 4°C at 40% power. Protein concentrations were measured by means of the Quantipro BCA assay kit (Sigma-Aldrich) according to the manufacturer's protocol. Absorbance was acquired using a microtiter plate reader (SpectraMax 340PC, Molecular Devices), and analysed using SOFTmax Pro3.1.1 software (Molecular Devices).

Caseinase and gelatinase activity assays

In order to investigate the enzymatic activity of plasmin (caseinase), we used a highly sensitive fluorescent based assay "Sensolyte® AFC Plasmin Activity Assay Kit" (AnaSpec, Fremont, CA, USA). In order to investigate the enzymatic activity of MMP-2/9 (gelatinase) we used a highly sensitive fluorescent based assay "EnzCheck® Gelatinase/ Collagenase Assay Kit" (Molecular Probes, Eugene, OR, USA). The caseinase and gelatinase assays were performed according to manufacturer's protocols, respectively.

Western blot analysis

For total and phosphorylated protein analyses, 2X SDS loading buffer was added to lysate samples containing equal amounts of protein (10 µg). These samples were heated for all protein analysis studies except for those involving ABCB1, for which samples were loaded without heating to avoid aggregation of these highly glycosylated transmembrane proteins. Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis, with primary antibodies diluted 1:1000 in 5% skim milk (Sigma-Aldrich) and 0.1 M tris-buffered saline–X-100 (TBS-T). The following antibodies were used: Rabbit anti-ABCB1 (sc-8313) and rabbit anti-claudin 5 (sc-28670) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Rabbit anti-total stress-activated protein kinases /Jun amino-terminal kinases (SAPK/JNK) (9252), anti-phopho SAPK/JNK (9251), anti-total p38 mitogen-activated protein kinase (MAPK) (9212) and anti-phospho-p38 MAPK (9211) were purchased from Cell Signaling Technology, Danvers, MA USA. Rabbit anti-LRP1 (ab92544) and anti-RAGE (ab37647) were obtained from Abcam Inc. Rabbit anti-occludin (71-1500) was purchased from Life Technologies Inc., and anti-β-actin (MAB1501) was purchased from EMD Millipore, Billerica, MA, USA. Primary antibodies were detected with horseradish peroxidase (HRP)–conjugated secondary immunoglobulin G (IgG) that were diluted 1:5000 in 5% skim milk and TBS-T and revealed by enhanced chemiluminescence plus (ECL) solution (GE Healthcare Life Sciences). Blots were digitized, densitometrically analyzed with ImageJ image analysis software (NIH), corrected for protein loading by means of β-actin, and expressed as relative values comparing control groups with treated groups.

Flow cytometry

Flow cytometry analysis was used to determine the population of monocytes in the circulation. Facial vein blood was collected in EDTA coated vials (Sarstedt, Newton, NC, USA). Flow

cytometry analysis was performed as described (Lampron et al., 2013). Briefly, 50 µL of total blood was incubated on ice for 15 minutes with 4 µL purified rat anti-mouse CD16/CD32 antibody (BD Bioscience) diluted in 35 µL DPBS. Always on ice, the mixture of cells and anti-mouse CD16/CD32 was incubated with Alexa Fluor® 700-conjugated anti-CD11b antibody (eBioscience), APC (allophycocyanin)-conjugated anti-CD115 antibody (eBioscience), V500-conjugated anti-CD45 antibody (BD BioScience), V450-conjugated anti-Ly6-C antibody (BD BioScience), and PE-congugated-Ly6G (BD Bioscience) for 45 minutes. Red blood cells were lysed with 1.5 mL Pharm Lyse buffer, according to manufacturer's protocol (BD BioScience). After hemolysis, remaining cells were washed with DPBS and resuspended in equal volumes of DPBS. Finally, the cells were analyzed using a LSR II flow cytometer and data acquisition was done with BD FACS Diva software (Version 6.1.2, BD Bioscience). The results were analyzed using FlowJo software (Version 7.6.1, Tree Star Inc.).

Behavior analysis

The T-water maze paradigm was used to assess mice the right-left discrimination learning of mice, in the weeks following the last injection (Filali and Lalonde, 2009). This paradigm evaluates the ability of mouse to remember the spatial location of a submerged platform. The T-maze apparatus (length of stem, 64 cm; length of arms, 30 cm; width, 12 cm; height of walls, 16 cm) was made of clear fiberglass and filled with water (23 ± 1 °C) at a height of 12 cm. An escape platform (11 × 11 cm) was placed at the end of the target arm and was submerged 1 cm below the surface. The position of the platform was chosen randomly for each animal prior to testing. In the acquisition-learning phase, which allows the evaluation of left/ right spatial learning, the mice were placed in the stem of the T-maze and swam freely until they found the submerged platform (located either in the right or in the left arm of the T-maze apparatus) and escaped to it. After reaching the platform, the mice remained on it for 20

seconds and then placed back in the maze for up to a maximum of 24 trials, except for a 10 minutes rest period after each 10 trial block. If the animals did not find the platform within 60 seconds, they were gently guided onto it. During the rest period, mice were dried with towels and provided with heating pads to prevent hypothermia. All trials were performed on one single day. A mouse was considered to have achieved criterion after 5 consecutive errorless trials. The reversal-learning phase was then conducted 2 days later, with the protocol repeated except that the mice were trained to find the escape platform on the opposite side. During the acquisition/reversal phase the platform was located in the same position for the entire stage. The number of trials to reach the criterion (5 of 5 correct choices made on consecutive trials) and the average of swimming speeds were recorded and analyzed.

Based on their scores during the reversal phase, the animals were subdivided in 3 subgroups (no cognitive deficit = as defined by values below 10 trials; mild cognitive deficit = as defined by values between 10-16 trials; severe cognitive decline = as defined by values above 16 trials).

In vitro **experiments**

Cell culture: The immortalized murine microglial cell line (BV2) were cultured at 37°C in 5% CO2, 95% air in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Inc.) containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. In all experiments, cells were grown to 70-90% confluence and subjected to a maximum of 20 cell passages.

Cell stimulation: BV2 ($6x10^5$ cells) were stimulated with 0,1 nM rt-PA (Roche), 2 µg/mL LPS or 5 ng/mL mIL-4 for 30 minutes at 37°C. Afterwards, cells were dissociated using a non-enzymatic cell dissociation buffer (Sigma-Aldrich), washed twice with PBS, and lysed in 1% NP-40 lysis buffer supplemented with 5% Protease Inhibitor Cocktail and 1% Phosphatase

Inhibitor Cocktail 2 (Sigma-Aldrich). Lysates were gently sonicated using the Sonic dismembrator model 100 (Fisher Scientific). Protein levels were quantified using the Quantipro BCA assay kit (Sigma-Aldrich). Cell lysates were used to investigate intracellular signaling pathways using Western blot or enzymatic activity using fluorescent based assay kits.

Cell migration assay: BV2 cells were seeded at $4x10^4$ (cells/ well) in a 24-well plate (BD Falcon®, BD, Mississauga, ON, Canada). Cell growth was stop by changing the media to DMEM / 1% FBS when the density was about 80 to 100%. Using a sterile tip, a wound was made in the middle of the well, directly afterwards cells were stimulated with 0,1 nM rt-PA (Roche), 0,1 nM rt-PA (S478A) (Abcam Inc.), 2 µg/mL LPS, 5 ng/mL mIL-4 and 0,1 nM rt-PA + 200 nM recombinant receptor-associated protein (RAP) (LRP1 inhibitor) (EMD Millipore). Pictures were acquired at 0 and 24 hours post-treatment using Olympus IX81 inverted research confocal microscope (Olympus America, Center Valley, PA, USA). Acquired images were analyzed using ImageJ image analysis software (NIH), where the number of infiltrated cells into the scratch was assessed.

Chemotaxis assay: Transwell polycarbonate 8 µm inserts (Corning, Lowell, MA) were coated with 2 µg/mL laminin (Sigma-Aldrich) overnight at 4°C. Inserts were equilibrated in DMEM / 1% FBS at least for 1 hour. BV2 cells were harvested and seeded at $4\text{-}5x10^4$ (cells/ well) with or without 200 nM RAP (EMD Millipore) that were added to the upper chamber. The lower chamber contained DMEM / 1% FBS with or without 0,1 nM rt-PA (Roche). The microplate was placed 24 hours in a humidified incubator at 37°C. Cells were dissociated using a non enzymatic dissociation buffer, collected from both compartments and counted with a hemacytometer.

Phagocytosis assay: BV2 cells $1x10^5$ (cells/ well) in 96-well microplate were stimulated with 0,1 nM rt-PA (Roche), 0,1 nM rt-PA (S478A) (Abcam Inc.), 1 µg/mL LPS or 5 ng/mL mIL-4 for

1 hour at 37°C. Stimulated cells were incubated with 1mg/mL fluorescein-labeled E. coli beads (Molecular Probes) for 2 hours at 37°C. Afterwards, cells were incubated with 250 µg/mL blue trypan (Molecular Probes) at room temperature for 1 minute. Phagocytosis rate was determined by measuring fluorescence emission at 520 nm following an excitation at 480 nm with a fluorescent plate reader SpectraMAX Gemini (Molecular Devices).

Griess Assay: Oxidative stress was quantified by measuring nitrite release in cell culture medium by using the Griess Assay according to manufacturer's protocol (Life Technologies Inc.). Briefly, BV2 cells were incubated for 24 hours with rt-PA (0,1 nM), LPS (1 µg/mL), IL-4 (5 ng/mL) or kept without stimulation. The cell culture medium was then transferred into a 96-well plate and mixed with 20 µL of Griess reagent and 130 µL of deionized water. In parallel, a standard curve was created ranging from 1 µM to 100 µM. The samples and standards were incubated for 30 minutes at room temperature. The nitrite concentrations were determined by measuring the absorbance at 548 nm by using a microtiter plate reader (SpectraMax 340PC, Molecular Devices), and analyzed using SOFTmax Pro3.1.1 software (Molecular Devices).

Oligomeric $A\beta_{1-42}$ Preparation and Internalization: Oligomeric HiLyte^{TM} Fluor 488-labeled human $A\beta_{1-42}$ (Anaspec) was prepared as described (Michaud et al., 2013). Briefly, labeled $A\beta_{1-42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma) to 1mM, dried under vacuum, and immediately dissolved in DMSO (Sigma) to 5 mM before use. Oligomeric $A\beta_{1-42}$ was prepared by adding ice-cold phenol red-free Ham's F-12 medium (Sigma) to obtain a final concentration of 100 µM. Afterwards, the solution rested for 48 hours at 4 °C. BV2 cells were plated on coverslips in six-well plates at a density of 1×10^6 cells/ml. Before the addition of oligomeric $A\beta_{1-42}$, BV2cells were stimulated for 1 h with 0,1 nM rt-PA (Roche). The cells were then incubated with 1 µg/mL of oligomeric HiLyte^{TM} Fluor 488-labeled human $A\beta_{1-42}$ in

a serum-deprived culture medium for 2 h at 37 °C. Cells were then washed three times with DPBS, stained for cytoskeleton (phalloidin) and nucleus (DAPI), and fixed with 4% PFA for 15 minutes at room temperature. Following fixation, cells were analyzed under laser scan confocal microscopy.

Supplementary Legend to Figures

Figure S1. BBB tightness is preserved after Activase® rt-PA administration. Microphotograph **(a)**, and **(b, c)** immunohistochemical analysis examining the purity of isolated cerebral microvessels, and BBB permeability to blood-borne molecules in the brain of APPswe/PS1 mice. The bright-field microphotograph **(a)** outlines the purity of isolated brain microvessels. The immunohistochemical analysis shows no **(b)** albumin and **(c)** IgG extravasation staining in the brain parenchyma of APPswe/PS1 mice 10 weeks after Activase® rt-PA weekly systemic administration. Images were acquired with a 40X objective. Scale bar = 50 µm **(a)**; 25 µm **(b, c)**.

Figure S2. BBB integrity is preserved after Activase® rt-PA administration. Western blot analysis, using brain capillary extracts from wildtype mice and examining the expression levels of proteins involved in BBB physical and functional proprieties, 3 **(a, c, e)** and 24 hours **(b, d, f)** after a single systemic Activase® rt-PA administration. The protein expression levels of **(a, b)** Occludin, **(c, d)** Claudin 5, and **(e, f)** ABCB1, do not change. Optical densities were corrected with β-actin levels. Data are means ± SEM (*n* = 4-6). MVs, microvessels.

Figure S3. Endothelial transporters involved in Aβ transport across the BBB are not affected following Activase® rt-PA administration. Western blot analysis, using brain microvessel extracts from wildtype mice and examining the expression levels of endothelial transporters involved in Aβ transport across the BBB, 3 **(a, c)** and 24 hours **(b, d)** after a single systemic Activase® rt-PA administration. The protein expression levels of **(a, b)** LRP1 and **(c, d)** RAGE, do not change. Optical densities were corrected with β-actin levels. Data are means ± SEM (*n* = 5-6). MVs, microvessels.

Figure S4. t-PA-associated perivascular proteases are not induced by Activase® rt-PA regimen. Caseinase **(a)**, gelatinase **(b)** activity assays examining plasmin (caseinase) and MMP2/9 (gelatinase) enzymatic activities in the brain and the microvasculature of APPswe/PS1 mice and their wildtype littermates. Chronic systemic Activase® rt-PA administration **(a)** does not change plasmin and **(c)** MMP2/9 activities in the brain and the microvasculature of APPswe/PS1 treated mice. In addition, the acute systemic Activase® rt-PA treatment **(b)** does not change the plasmin and **(d)** MMP2/9 activities in the brain and the microvasculature of wildtype mice 3 hours after injection. Data are means ± SEM (*n* = 6-8). MVs, microvessels; TBH, total brain homogenates; WT, wildtype.

Figure S5. Chronic Activase® rt-PA administration modulates monocyte subpopulation frequencies in the blood of APPswe/PS1 mice. Flow cytometry analysis **(a-c)** was performed to examine total monocyte population frequency and subset frequencies in the blood of APPswe/PS1 mice. Activase® rt-PA **(a)** does not change total monocyte frequency in leukocytes (CD45+ cells) in the blood 24 hours after last injection. **(b)** A gating strategy was thereafter used to discriminate between inflammatory monocyte ($Ly6C^{high}$) and patrolling monocyte ($Ly6C^{low}$) subset frequencies in the total population of monocytes. Activase® rt-PA **(c)** significantly decreases $Ly6C^{high}$ monocyte subset frequency, without **(d)** modulating $Ly6C^{low}$ subset frequency in the blood 24 hours after last injection. Data are means ± SEM (*n* = 10 animals per group). * $P < 0.05$ compared with saline treated group (standard two-tailed unpaired *t*-test's).

Figure S6. Acute Activase® rt-PA administration modulates total monocyte frequency in the blood of APPswe/PS1 mice. Flow cytometry analysis was performed to examine total monocyte population frequency and subset frequencies in the blood of APPswe/PS1 mice.

Activase® rt-PA **(a)** increases total monocyte frequency in leukocytes (CD45+ cells) in the blood 3 hours after injection. However, Activase® rt-PA **(b)** does not modulate Ly6C^{high} monocyte subset frequency and **(c)** Ly6C^{low} subset frequency in the blood 3 hours after injection. Data are means ± SEM (*n* = 7-8 animals per group). * *P* < 0.05 compared with saline treated group (standard two-tailed unpaired *t*-test's).

Figure S7. Acute Activase® rt-PA administration modulates monocyte subpopulation frequencies in the blood of wildtype mice. Flow cytometry analysis **(a-f)** examining total monocyte population frequency and subset frequencies in the blood of wildtype mice 3 **(a, c, e)** and 24 hours **(b, d, f)**, following Activase® rt-PA administration. rt-PA **(a)** does not change the total monocyte frequency in circulating leukocytes (CD45+ cells) 3 and **(b)** 24 hours after a single systemic injection in wildtype mice. This treatment also failed to affect **(c)** Ly6C^{high} monocyte subset frequency in the blood 3 and **(d)** 24 hours after a single systemic injection. However, it **(e)** significantly increases Ly6C^{low} subset frequency 3 hours, but not **(f)** 24 hours after injection. Data are means ± SEM (*n* = 6-7 animals per group) * *P* < 0.05 compared with saline treated group (standard two-tailed unpaired *t*-test's).

Figure S8. Activase® rt-PA administration does not influence blood-derived monocyte infiltration into the brain parenchyma of APPswe/PS1 mice. Immunofluorescence staining **(a, c)** and flow cytometry analysis **(b)** examining blood-derived monocyte infiltration into the brain parenchyma of APPswe/PS1. Double Iba1/CD45 immunofluorescence staining shows **(a)** the absence of CD45^{high}/Iba1 positive cells (blood-derived macrophages) surrounding Aβ plaques in the brain of APPswe/PS1 mice, 10 weeks after systemic Activase® rt-PA administration. Flow cytometry analysis shows **(b)** that the frequencies of CD11b^{high}/CD45^{high} cells (blood-derived macrophages) remain unchanged in the brain of APPswe/PS1 mice, 3

and 24 hours following a single Activase® rt-PA administration. Finally, Activase® rt-PA **(b)** does not trigger the infiltration of blood-borne GFP-positive cells into the brain of APPswe/PS1 chimeric mice 24 hours after a single rt-PA administration. In contrast, irradiation **(c)** triggers the infiltration and differentiation (ramification) of blood-borne GFP-positive cells in the brain of APPswe/PS1 mice (positive control). Data are means ± SEM (*n* = 6-7 animals per group, 4 sections representing the rostral, middle and caudal levels of the hippocampus and overlaying cortex per animal's). Scale bar = 50 µm **(a)**; 250 µm **(c)**.

Figure S9. Chronic Activase® rt-PA administration does not trigger a sustained inflammation in the brain of chimeric APPswe/PS1 mice. *In situ* hybridization examining IkBα expression, an indicator of NF-kB activity, following Activase® rt-PA administration. The acute administration of rt-PA **(a)** does not induce NF-kB activation, which is assessed by the expression levels of IkBα gene transcript. Data are means ± SEM (*n* = 3-4). * $P < 0.05$ compared with saline treated group (standard two-tailed unpaired *t*-test's). Brain sections of mice systemically injected with LPS were used as positive controls for NF-kB activation. Images were acquired with a 4X objective. Scale bar = 250 µm.

Figure S10. rt-PA stimulation enhances oligomeric $A\beta_{1-42}$ internalization by BV2 microglial cells. Immunofluorescence staining examining the internalization of oligomeric HiLyte^{TM} Fluor 488-labeled human $A\beta_{1-42}$ by BV2. rt-PA enhances the internalization of fluorescent $A\beta_{1-42}$ (green) into BV2 microglial cells, which were counterstained with phalloidin (red) and DAPI (blue). Laser scan confocal images were acquired with a 40X objective. Scale bar = 10 µm.

Figure S1

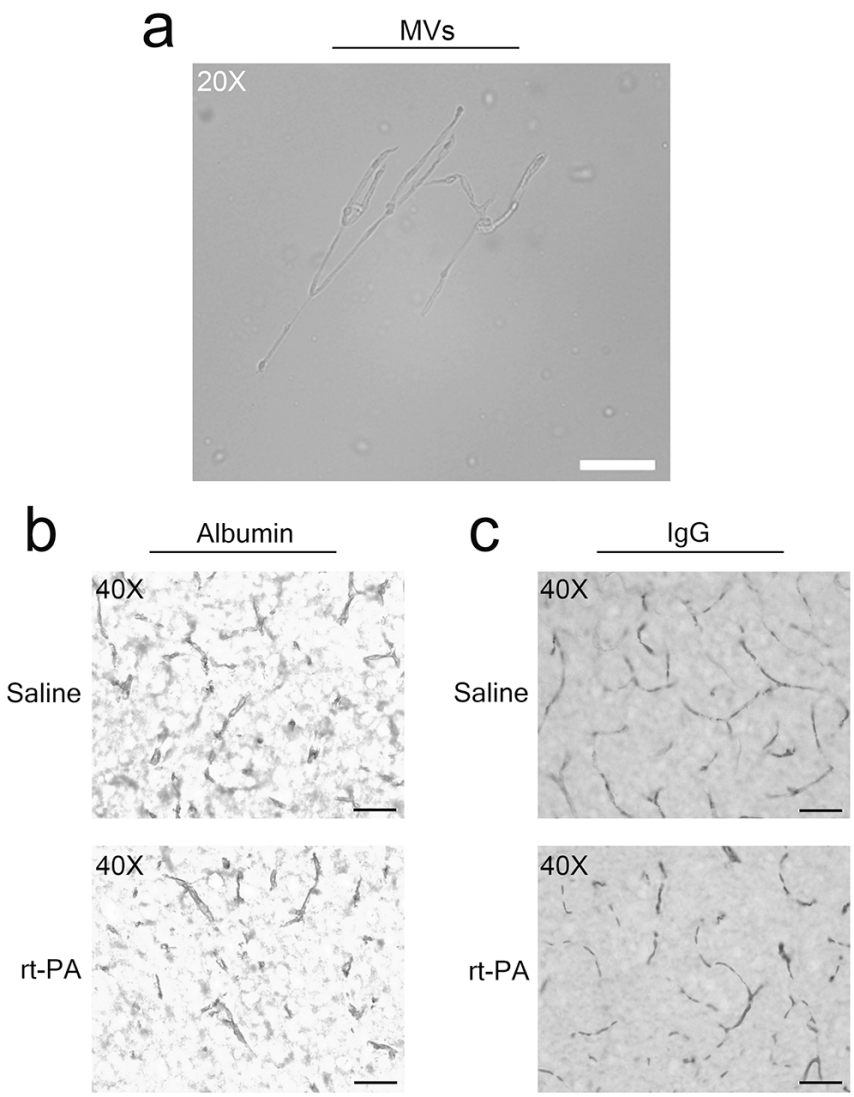


Figure S2

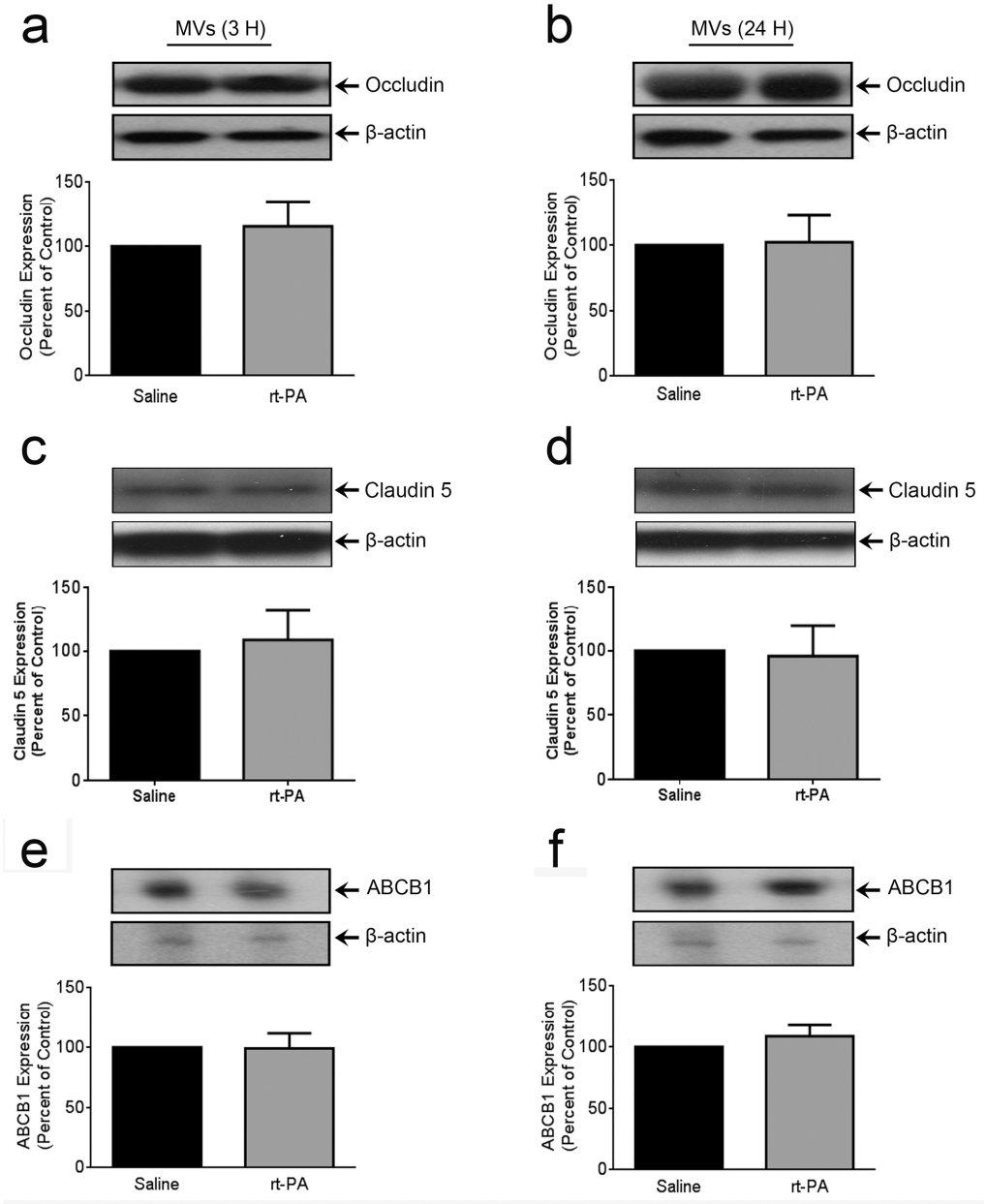

a
MVs (3 H)
Occludin
β-actin
Occludin Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
b
MVs (24 H)
Occludin
β-actin
Occludin Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
c
Claudin 5
β-actin
Claudin 5 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
d
Claudin 5
β-actin
Claudin 5 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
e
ABCB1
β-actin
ABCB1 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
f
ABCB1
β-actin
ABCB1 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA

Figure S3

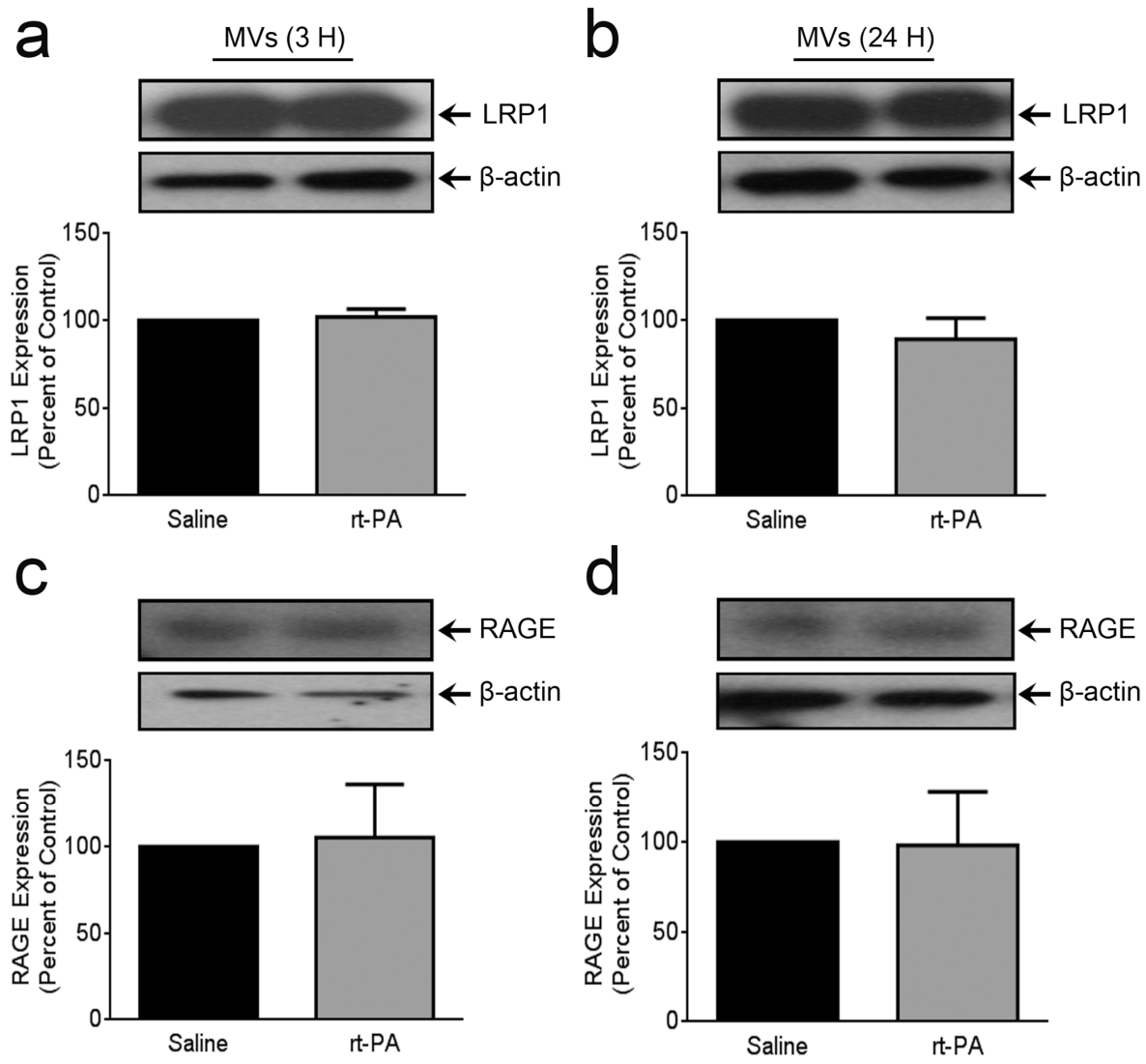

a
MVs (3 H)
LRP1
β-actin
LRP1 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
b
MVs (24 H)
LRP1
β-actin
LRP1 Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
c
RAGE
β-actin
RAGE Expression (Percent of Control)
150
100
50
0
Saline
rt-PA
d
RAGE
β-actin
RAGE Expression (Percent of Control)
150
100
50
0
Saline
rt-PA

Figure S4

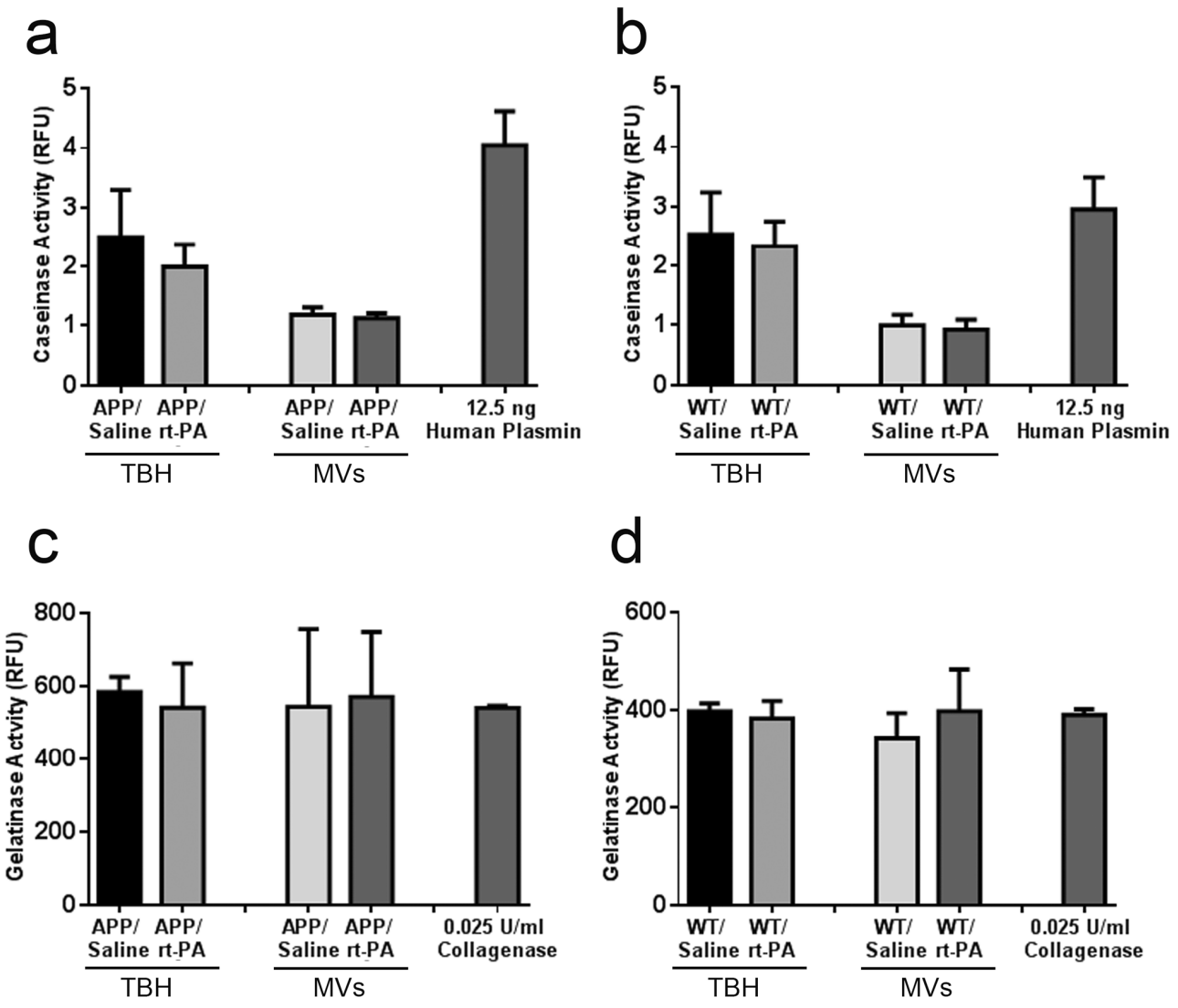

a
Caseinase Activity (RFU)
0
1
2
3
4
5
APP/ Saline
APP/ rt-PA
APP/ Saline
APP/ rt-PA
12.5 ng Human Plasmin
TBH
MVs
b
Caseinase Activity (RFU)
0
1
2
3
4
5
WT/ Saline
WT/ rt-PA
WT/ Saline
WT/ rt-PA
12.5 ng Human Plasmin
TBH
MVs
c
Gelatinase Actvity (RFU)
0
200
400
600
800
APP/ Saline
APP/ rt-PA
APP/ Saline
APP/ rt-PA
0.025 U/ml Collagenase
TBH
MVs
d
Gelatinase Actvity (RFU)
0
200
400
600
WT/ Saline
WT/ rt-PA
WT/ Saline
WT/ rt-PA
0.025 U/ml Collagenase
TBH
MVs

Figure S5

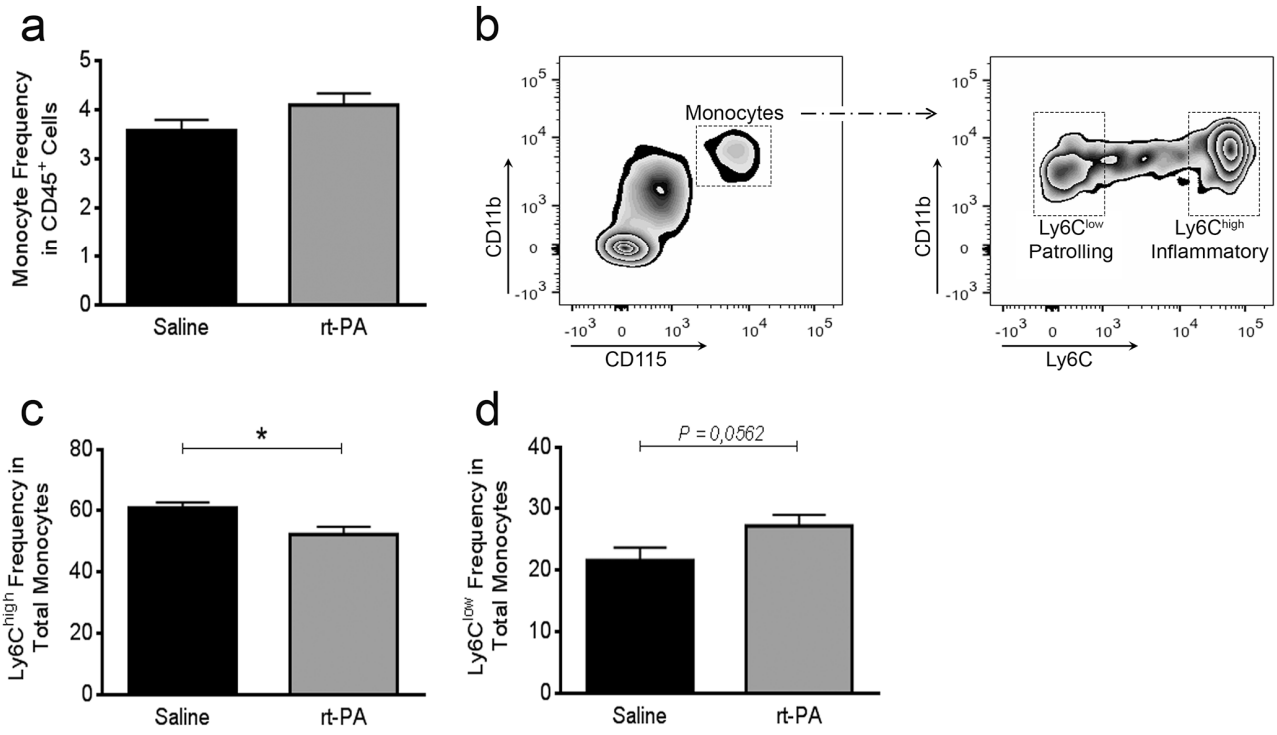

a
Monocyte Frequency in CD45+ Cells
Saline
rt-PA
b
Monocytes
CD11b
CD115
Ly6C
Ly6Clow Patrolling
Ly6Chigh Inflammatory
c
Ly6Chigh Frequency in Total Monocytes
*
Saline
rt-PA
d
Ly6Clow Frequency in Total Monocytes
P = 0,0562
Saline
rt-PA

Figure S6

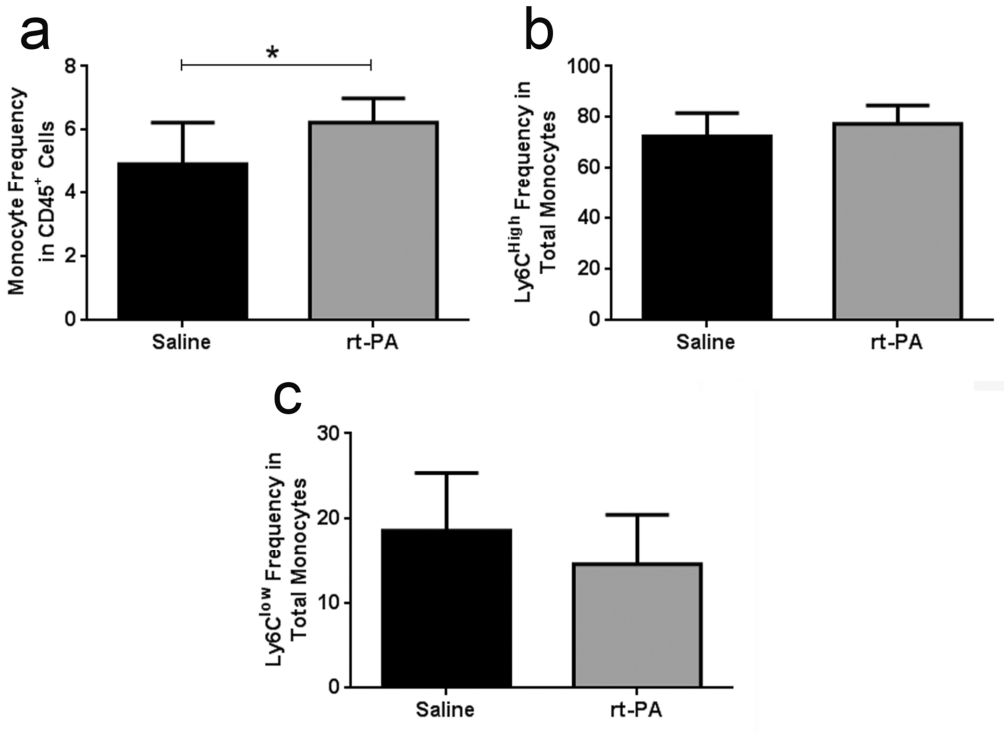

a
*
Monocyte Frequency in CD45+ Cells
0
2
4
6
8
Saline
rt-PA
b
Ly6CHigh Frequency in Total Monocytes
0
20
40
60
80
100
Saline
rt-PA
c
Ly6CLow Frequency in Total Monocytes
0
10
20
30
Saline
rt-PA

Figure S7

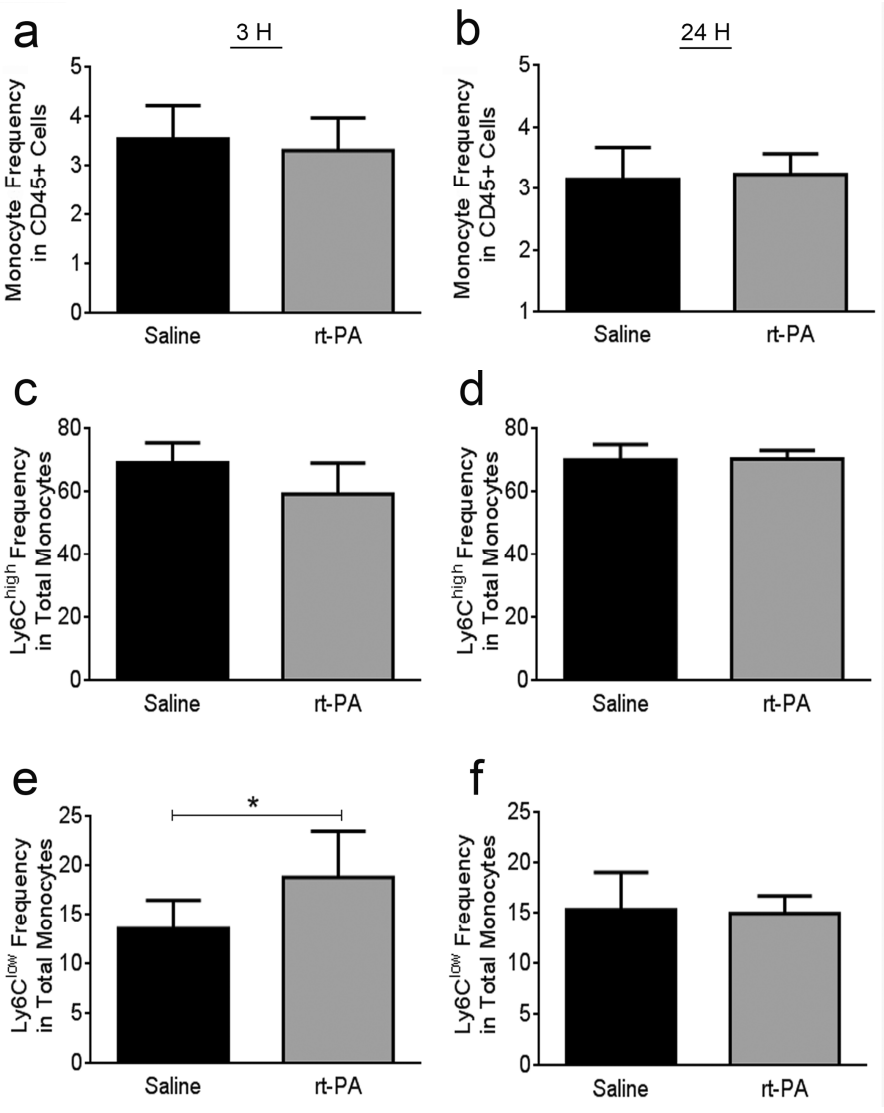

a
3 H
Monocyte Frequency in CD45+ Cells
Saline
rt-PA
b
24 H
Monocyte Frequency in CD45+ Cells
Saline
rt-PA
c
Ly6C^high Frequency in Total Monocytes
Saline
rt-PA
d
Ly6C^high Frequency in Total Monocytes
Saline
rt-PA
e
*
Ly6C^low Frequency in Total Monocytes
Saline
rt-PA
f
Ly6C^low Frequency in Total Monocytes
Saline
rt-PA

Figure S8

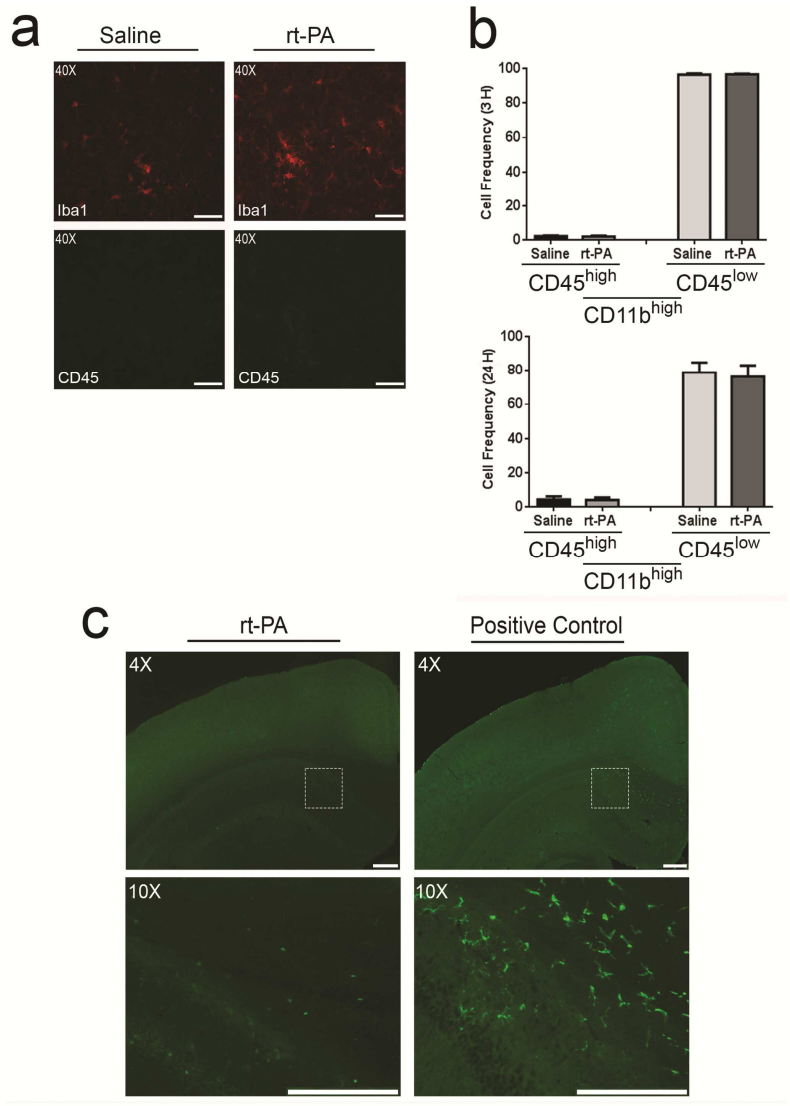

a
Saline
rt-PA
40X
Iba1
CD45
b
Cell Frequency (3 H)
Cell Frequency (24 H)
Saline
rt-PA
CD45high
CD45low
CD11bhigh
c
rt-PA
Positive Control
4X
10X

Figure S9

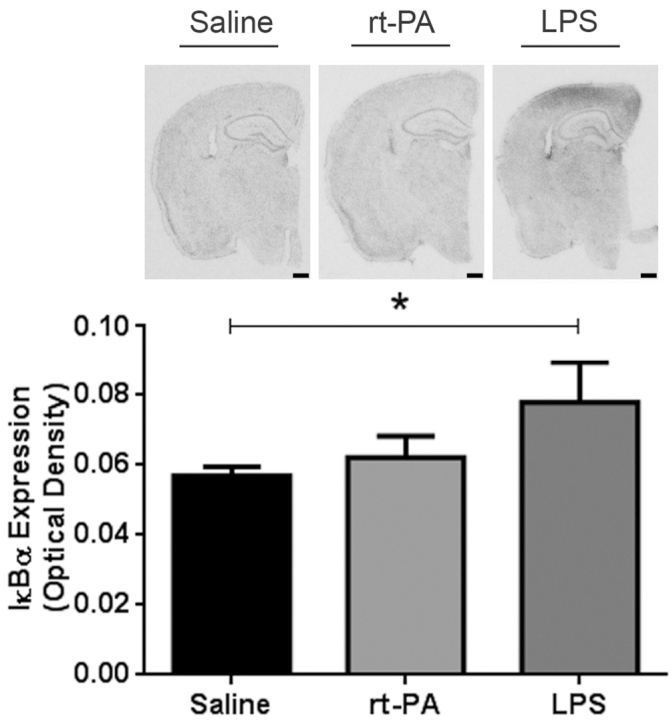
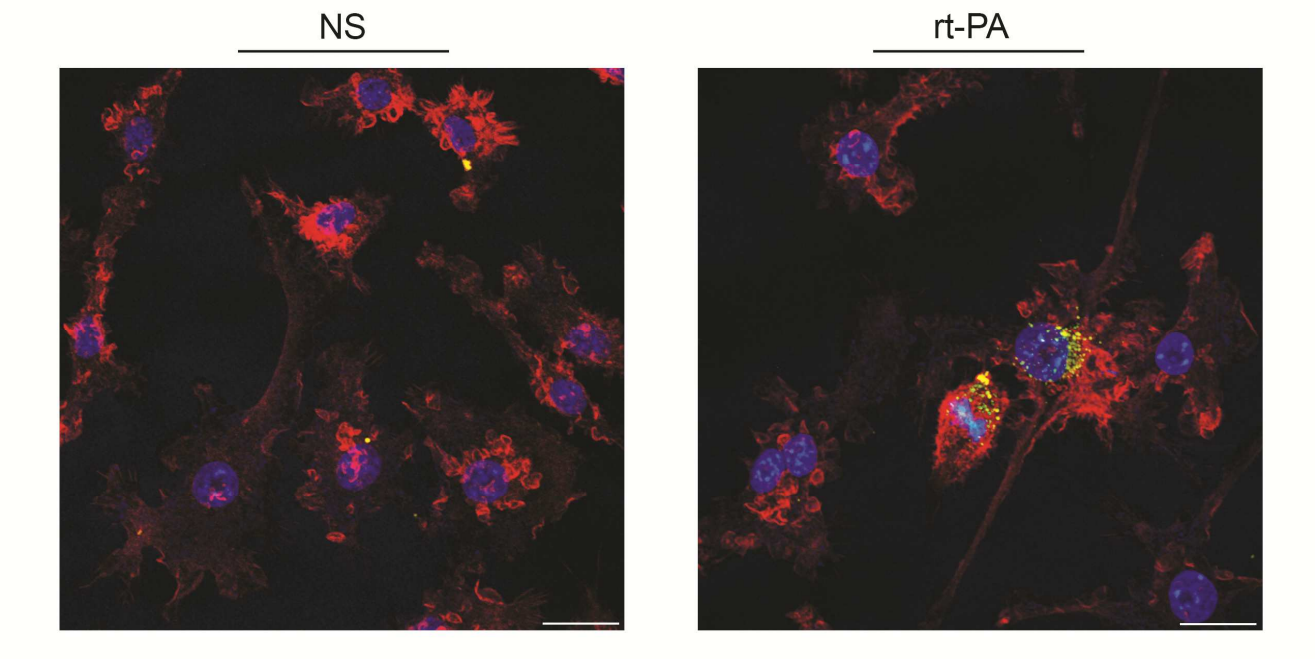

Saline
rt-PA
LPS
*
0.10
0.08
0.06
0.04
0.02
0.00
IκBα Expression
(Optical Density)
Saline
rt-PA
LPS

Figure S10



Supplementary Conclusion

It has been shown that a defective production of monocytes was accompanied by an increased accumulation of soluble Aβ in the brain of APPswe/PS1 mice, which was closely associated to the onset of cognitive decline (Naert and Rivest, 2012). Macrophage-colony stimulating factor (m-CSF) treatment prevented and restored the cognitive decline of mice by increasing total monocyte population frequency in blood circulation (Naert and Rivest, 2012). In parallel, microglia have been shown to be recruited to Aβ plaques, and to internalize and degrade Aβ microaggegates (Simard et al., 2006). However, over time, the capacity of resident microglia to clear Aβ decreases and becomes inefficacious due to the presence of an exacerbated pro-inflammatory microenvironment (Hickman et al., 2008). Nonetheless, the immunostimulation of resident microglial cells by exogenous agents, such as the detoxified TLR4-ligand monophosphoryl lipid A (MPL), reinforced Aβ clearance by microglial cells and improved the cognitive functions of APPswe/PS1 mice (Michaud et al., 2013). These observations suggest that the therapeutic strategies that aim at boosting monocyte production and microglial cell phagocytic activity with moderate inflammatory responses are very promising, and constitute attractive avenues in combating AD progression. Therefore, our study suggests that the Activase® rt-PA might act as an immunomodulatory molecule that can modulate monocyte and microglial cell functions.

Supplementary References

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Hickman SE, Allison EK, El Khoury J (2008) Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci 28:8354-8360.

Laflamme N, Lacroix S, Rivest S (1999) An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood-brain barrier in response to a systemic and localized inflammation but not during endotoxemia. J Neurosci 19:10923-30.

Lampron A, Pimentel-Coelho PM, Rivest S (2013) Migration of bone marrow-derived cells into the central nervous system in models of neurodegeneration. J Comp Neurol 521:3863-3876.

Michaud JP, Hallé M, Lampron A, Thériault P, Préfontaine P, Filali M, et al (2013) Toll-like receptor 4 stimulation with the detoxified ligand monophosphoryl lipid A improves Alzheimer's disease-related pathology. Proc Natl Acad Sci U S A 110:1941-1946.

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Naert G, Rivest S (2012) Age-related changes in synaptic markers and monocyte subsets link the cognitive decline of APPSwe/PS1 mice. Front Cell Neurosci 6:51.

Simard AR, Soulet D, Gowing G, Julien JP, Rivest S (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 49:489-502.