Electronic Supplementary Material (ESI) for Nanoscale.

Hybrid Multimodal Contrast Agent for Multiscale *In Vivo* Investigation of Neuroinflammation

Supporting Material

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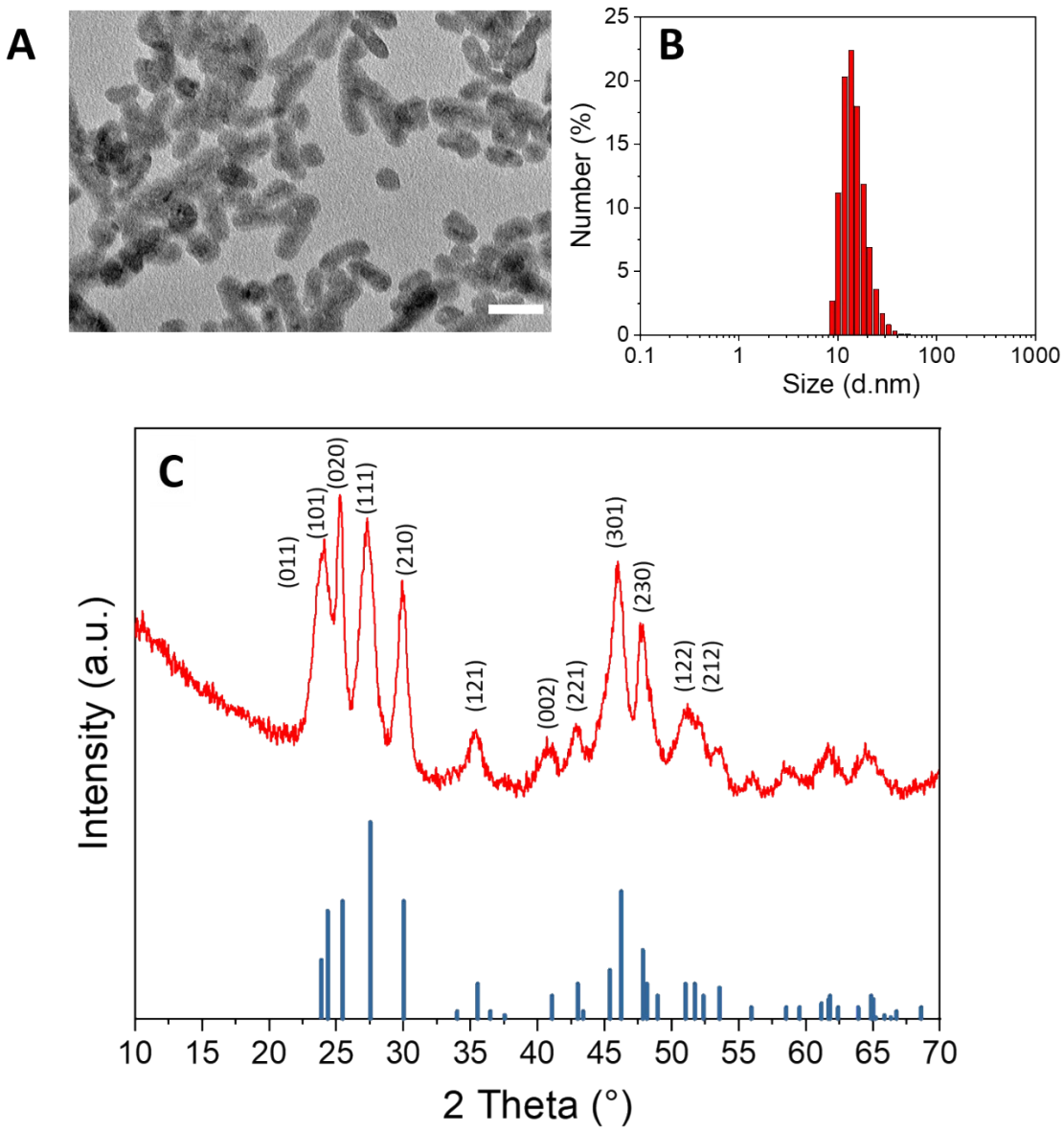

Fig. S1 Characterization of GdF_3 nanoparticles (**NP1**). **A)** TEM image of **NP1** particles. Scale bar represents 20 nm. **B) NP1** particle size determination by DLS measurement (mean size distribution by number is 16±5 nm with a PDI of 0.16) **C)** Measured XRPD pattern of **NP1** particles (in red) compared to the standard pattern JCPDS 12-0788 (in blue) corresponding to the orthorhombic phase (*Pnma* space group) of GdF_3.

Table S1 Elemental analysis results from XPS spectra of **NP1** and **NP3** nanoparticles

	C	O	Gd	F	N	P	Si	Na	Cl
NP1	-	6.0	67.7	26.3	-	-	-	-	-
NP3	12.2	13.7	41.3	24.7	-	2.2	-	6.0	12.2

Proportion of each element is reported in atomic % (at%)

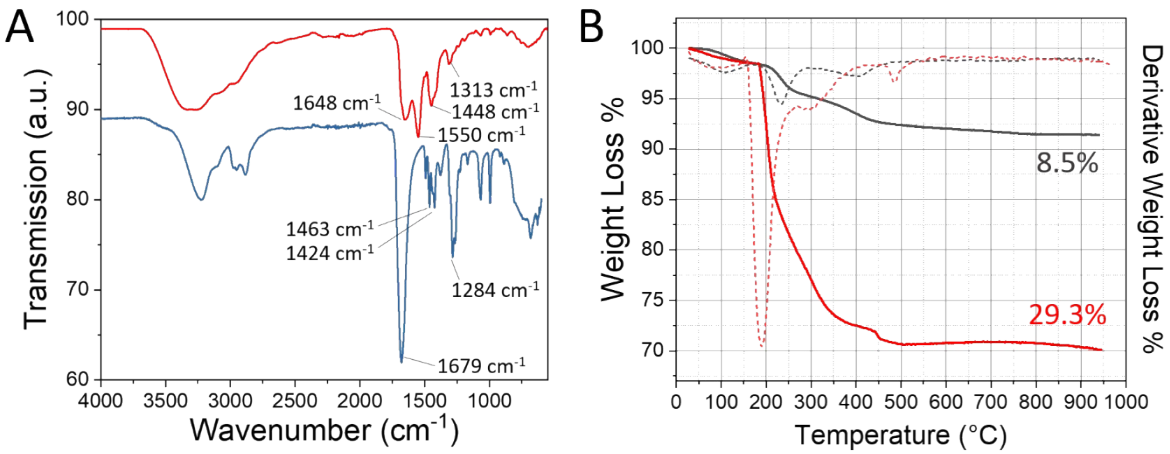

Fig. S2 A) FTIR-ATR spectrum of **NP1** (red line) compared with the spectrum of the solvent 2-pyrrolidinone (blue line). The open form of the solvent molecule is coordinating to the particle surface, which induces the reorganization of its vibrational bands. The solvent molecule coordination results in morphology control by limiting particle growth. **B)** TGA measurements. Solid black and red lines correspond to the weight loss (%) measured for **NP1** and **NP2** respectively. Dashed black and red lines show the derivative weight loss (%) for **NP1** and **NP2**, respectively.

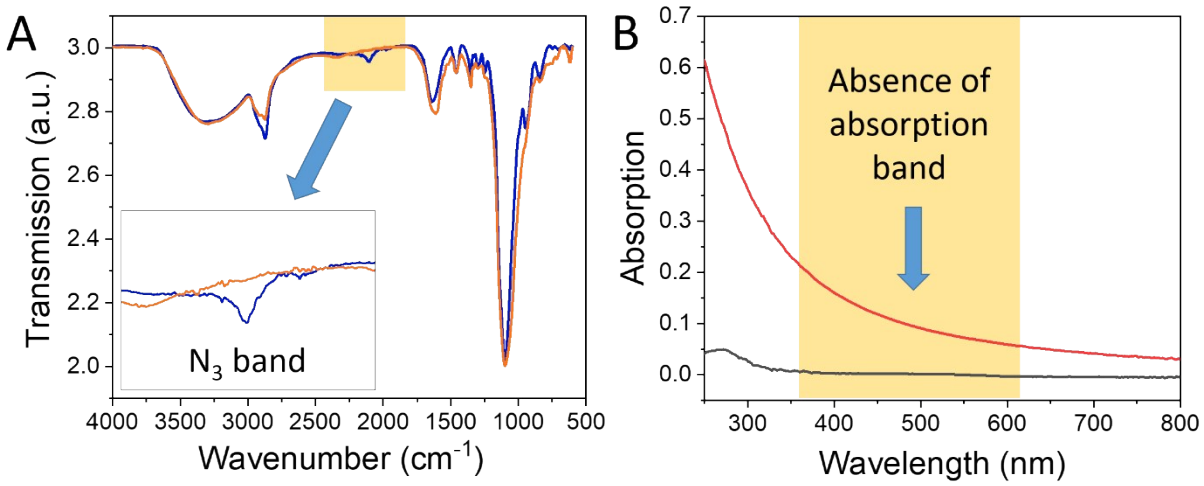

Fig. S3 A) FTIR-ATR spectrum of **NP2** (blue spectrum; nanoparticles after surface modification with bisphosphonate-PEG ligands) and **NP3** nanoparticles (orange spectrum; pegylated nanoparticles after coupling of the fluorophore **3**). Comparison of the two spectra shows the vanishing of the band at 2100 cm^{-1} after the coupling reaction, which supports reaction with the chromophore. B) **NP2** is incubated 20h with fluorophore **3** in the same conditions as for the fluorophore coupling reaction, but without heating. The same purification procedure is followed for these particles, as for the normal coupling reaction. The last supernatant (4^{th}) in methanol (black line) does not present the absorption band characteristic for **3** and the spectrum of the purified **NP2** nanoparticles shows only the scattering profile of the particles. This is consistent with the fact that compound **3** do not react without heating and in the system **NP3** the fluorophore is not entrapped in the PEG chains but is chemically attached.

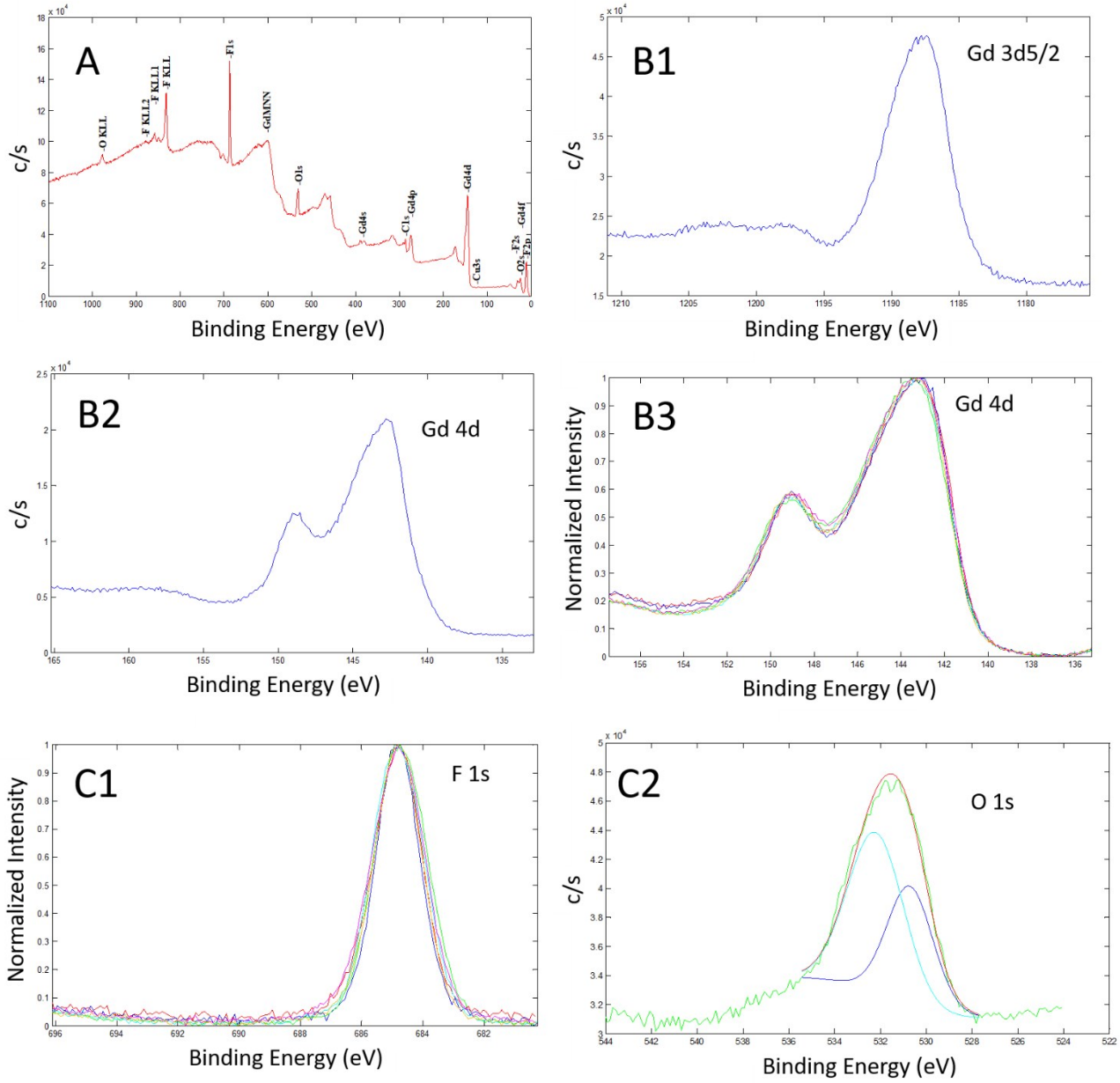

Fig. S4 XPS elemental analysis **A)** XPS survey spectrum of mineralized (*i.e.* subjected to pyrolysis for 5h at 700°C) **NP1**. **B1-3)** High resolution XPS spectra centered on Gd 4d (143.4 eV) and Gd 3d 5/2 (1187.8 eV) of **NP1** (B1, B2) and **NP3** (B3) nanoparticles. **C1,2)** High resolution XPS spectra centered on F 1s (684.8 eV) and on O 1s of **NP3** particle. The peak O 1s was decomposed to two contributions with peak maxima at 532.21 eV (61.11%) and 530.70 eV (38.89%) attributed to O-Gd and O-P binding energies respectively.

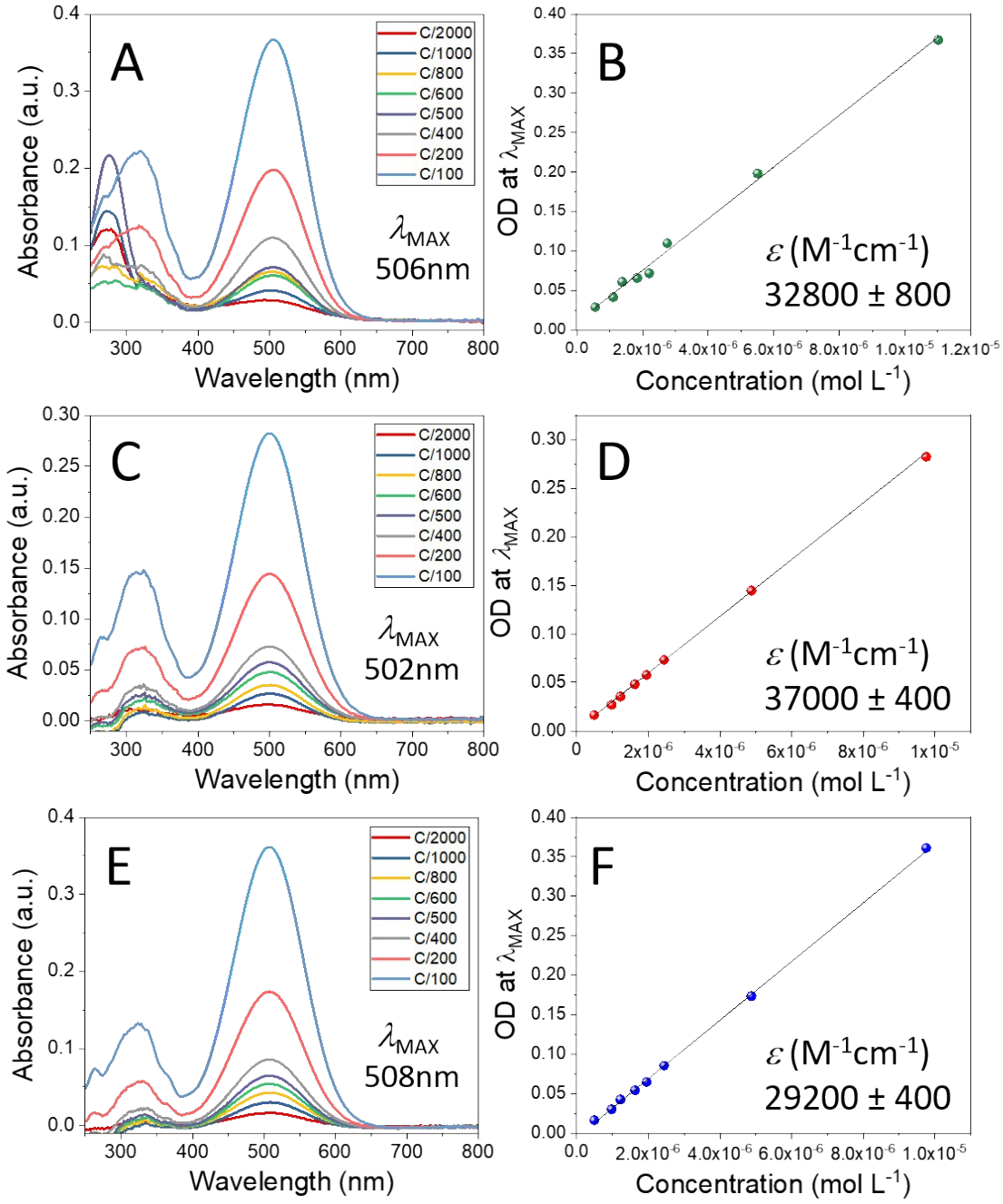

Fig. S5 Absorption spectra of compound **3** at different concentrations in dichloromethane **A**), in acetonitrile **C**) and in methanol **E**). Peak maxima are at 506 nm, 502 nm and 508 nm respectively. The initial concentration *C* (indicated in the legends) is $1.10 \cdot 10^{-3}$ mol L^{-1} in dichloromethane, $9.76 \cdot 10^{-4}$ mol L^{-1} in acetonitrile and $9.76 \cdot 10^{-4}$ mol L^{-1} in methanol solutions. Graphs **B**, **D** and **F** show the linear relationship of absorbance *vs* concentration used for determination of the molar absorption coefficient (ε) in dichloromethane **B**), in acetonitrile **D**) and in methanol **F**).

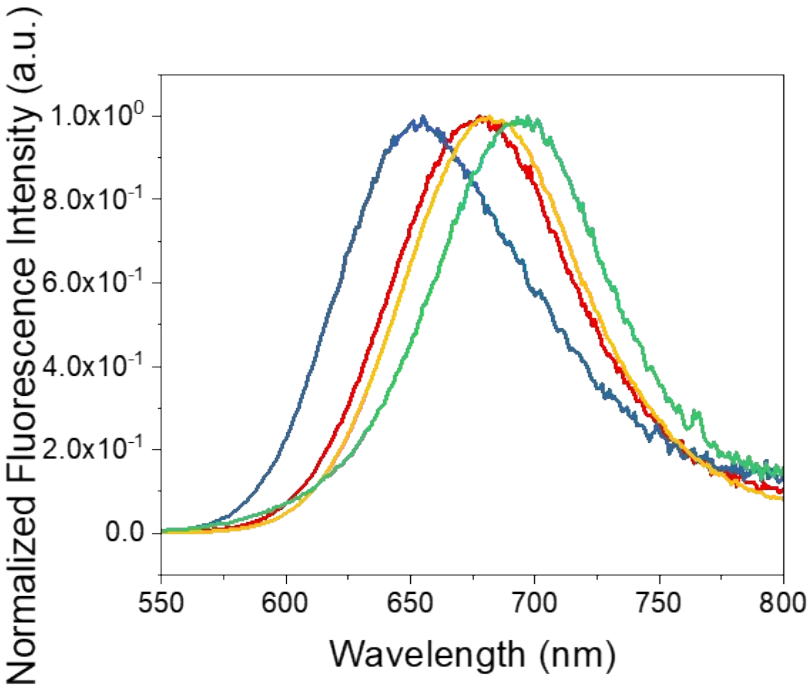

Fig. S6 Positive solvatochromism observed for the fluorescence emission spectra of free dye **3** in dichloromethane (blue), in acetonitrile (red) and in methanol (yellow). Upon excitation at 510 nm the emission maxima of these spectra are 634, 653 and 658 nm respectively. The emission spectrum of **NP3** aqueous suspension (green) is undergoing the most important solvatochromism, with a maximum emission at 682 nm.

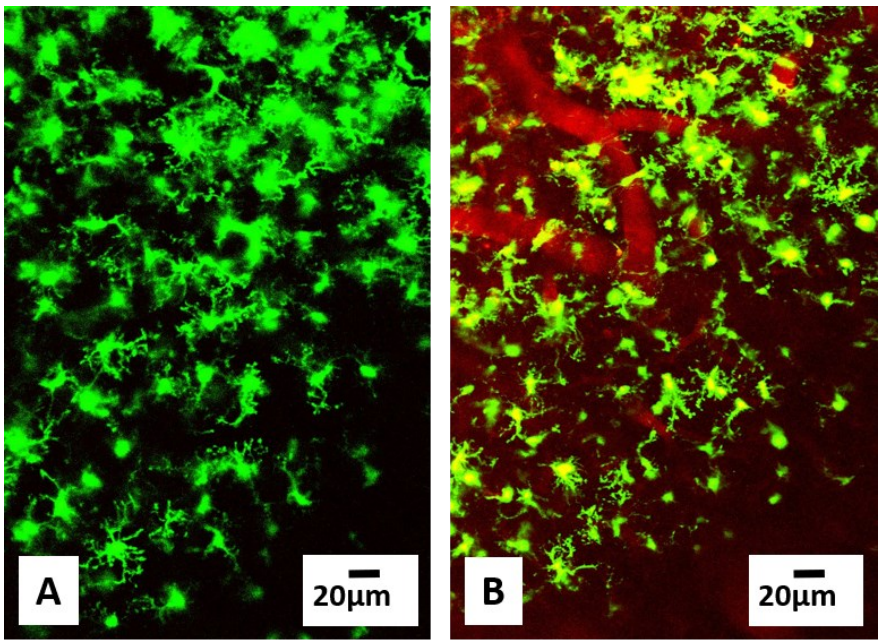

Fig S7. **NP3** vascular remanence observed by TPEM. *In vivo* two-photon images of a CX3CR1-GFP/+ mouse submitted to pMCAo. **A)** Representative image of round-shaped CX3CR1-GFP/+ cells in the ischemic lesion before **NP3** injection. **B)** The same area is observed 24h after **NP3** injection.

Bone-marrow derived macrophages (BMDM) culture

BMDM were generated from bone marrow (BM) extracted from 6-8 weeks old C57BL/6 mice. BM cells were isolated by flushing from the tibia and femurs. Erythrocytes were removed by RBC lysis buffer, and the remaining cells were cultured in a complete DMEM medium with 20% L929 conditioned medium (containing macrophage-colony stimulating factor) for 7 days in a 10 cm bacteriological plate. After 7 days, BMDM were detached by treating with accutase for 5 min (Biowest, L0950, Nuaille, France) and replated according to the assay type.

Immunocytochemistry

In order to evaluate **NP3** internalization by BMDM, 0.5 x 10^5 BMDM/ml were seeded in a 4-well Lab-Tek plate and incubated without **NP3** or either 0.5 mM or 1.5 mM **NP3** at 37°C with 5% CO_2. After 24 h, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). Plasma membranes were labelled by incubating the cells with FITC conjugated cholera toxin (20µg/ml; Sigma, C1655, Lyon, France) for 30 min. Cells were then washed with PBS and each chamber of the Lab-Tek plate was refilled with PBS. Finally, cellular preparations were imaged by confocal spinning disk microscope (Andromeda, TILL-FEI, Grenoble, France). After acquisition, images were processed with Icy 2.0.3.0 (open source image processing software).[1]

Flow cytometry

To quantify **NP3** internalization by BMDM, BMDM not exposed to **NP3** or exposed to 0.5 mM or 1.5 mM **NP3** were cultured in a 12-well plate for 24 hours at 37°C, with 5% CO_2. Then, the cells were labelled with antibodies specific for CD11b (Ozyme, BLE101216, Paris, France) and F4/80 (Ozyme, BLE123152, Paris, France) BMDM cell surface markers after blocking the Fc receptor (BD Pharmingen, 553142, Paris, France) to reduce nonspecific binding. Finally, cells were analyzed by flow cytometry using LSR II (Becton-Dickinson, Franklin Lakes, USA), and the proportion of **NP3** positive cells were quantified using FCS Express V5 (De Novo Software, Pasadena, USA).

Cytokine immunoassays

For measurement of interleukine-6 (IL-6) and tumor-necrosis factor-α (TNF-α), supernatants of **NP3**-exposed BMDM were extracted after 48 h of culture. As a positive control for IL-6 and TNF-α production, BMDM non-exposed to **NP3** were cultured during 24 h and then stimulated using 2 µg/mL lipopolysaccharide (LPS, Sigma, L2630, Lyon, France) harvested from E. Coli. Cultures were incubated for 24 h at 37°C with 5% CO_2. Finally, cytokine production was measured in the supernatant of cell

cultures using mouse IL-6 (ThermoFisher, KMC0061, Waltham, USA) and TNFα (ThermoFisher, BMS607-3, Waltham, USA) ELISA kit according to manufacturer's protocol.

Measurement of Nitric oxide (NO) production

For the assessment of NO production, BMDM were cultured in the same conditions and timeline as for the cytokine immunoassay. NO produced by BMDM was determined by measuring nitrite concentration in cell culture media by Griess assay. Briefly, 50 µL of cell supernatant was transferred to a 96-well plate and incubated with equal volume of Sulphanilamide (Sigma, S9251, Lyon, France) and N-alpha-naphthyl-ethylenediamine (NED) (Sigma, 222488, Lyon, France) solution respectively, and were allowed to sit for 10 min each, protected from light. Then, the optical density (OD) was measured at 540 nm using CLARIOstar® Microplate Reader (BMG LABTECH, Champigny-sur-Marne, France). The approximate concentration of nitrite in the samples was determined from a standard curve.

Lactate dehydrogenase (LDH) assay

BMDM were cultured without **NP3** or 0. mM and 1.5 mM **NP3** in a 12 well plate for 24 h at 37°C, with 5% CO_2. After that, the toxicity was measured by quantifying the cell viability using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, G7891, Charbonnières-les-Bains, France) according to the manufacturer's optimized protocol. Briefly, the protocol is the following: lysis solution (2 µl of Lysis Solution per 100 µl original volume) was used to generate a maximum lactate dehydrogenase (LDH) release, which was used as a positive control for cellular toxicity. To further prepare the test sample, an equal volume of CytoTox-ONE™ reagent (100 µL) was added to each well and the plate was placed on a shaker for 30 seconds and then incubated for another 10 minutes in the dark. After that, ½ of the volume of CytoTox-ONE™ Reagent (50 µL) was added to each well and the plate was placed on the shaker for another 10 seconds. Finally, the fluorescence was recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm using CLARIOstar® Microplate Reader (BMG LABTECH, Champigny-sur-Marne, France).

Statistical analysis

For BMDM culture quantitative analyses, significant differences were calculated using a one-way analysis of variance (ANOVA; R statistical software version 3.3.3 (2017–03–06) "Another Canoe."). In all comparisons, a p-value <0.05 was considered statistically significant. Data were represented as box plots (median [25% percentile; 75% percentile]).

1 F. de Chaumont, S. Dallongeville, N. Chenouard, N. Hervé, S. Pop, T. Provoost, V. Meas-Yedid, P. Pankajakshan, T. Lecomte, Y. Le Montagner, T. Lagache, A. Dufour and J.-C. Olivo-Marin, *Nat. Methods*, 2012, **9**, 690–696.