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Supplemental Information

TLR4-Binding DNA Aptamers Show a Protective Effect against Acute Stroke in Animal Models

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Supplementary Materials

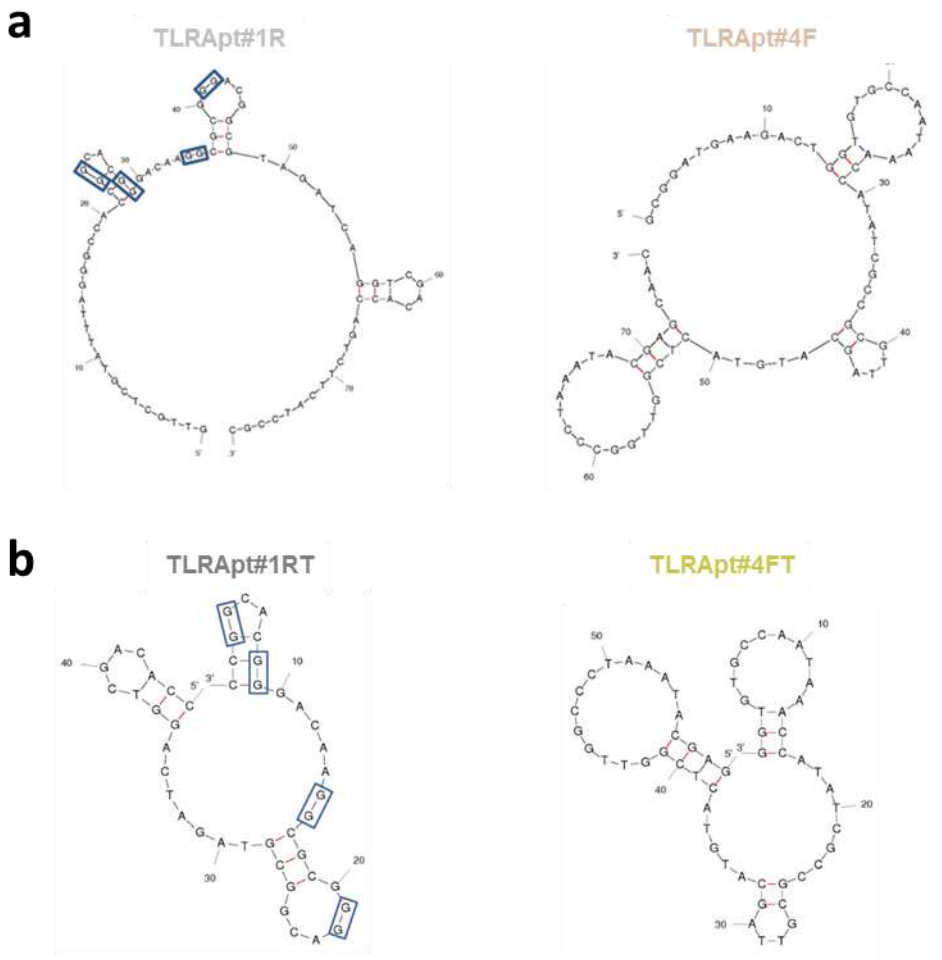

Figure S1. Predicting secondary structures of the parental aptamers TLRApt#1R and TLRApt#4F (**a**) and the truncated aptamers TLRApt#1RT and TLRApt#4FT (**b**) modelated using mFold software. The blue boxes indicate the guanines that potentially can form G-quadruplex structures.

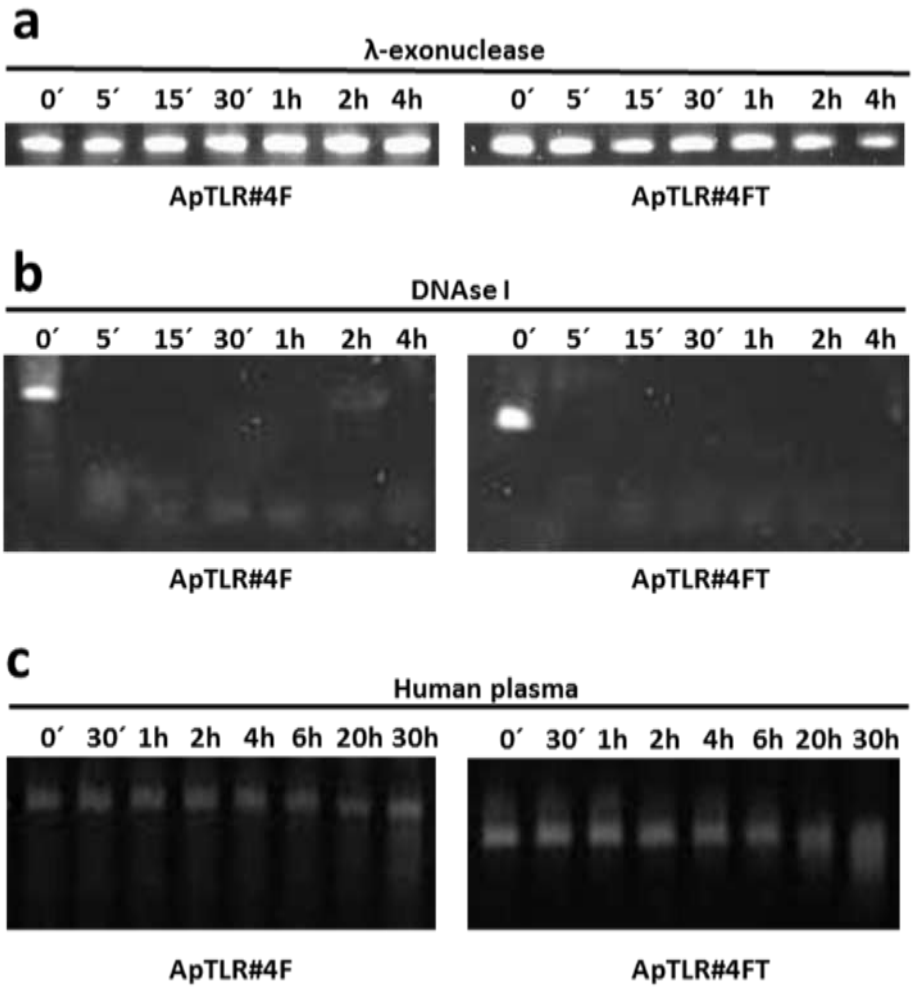

Figure S2. Resistance of ApTLR#4F and ApTLR#4FT to degradation by λ-exonuclease (**a**), DNAse I (**b**) and in human plasma (**c**) at 37°C. A representative gel from 3 experiments is shown.

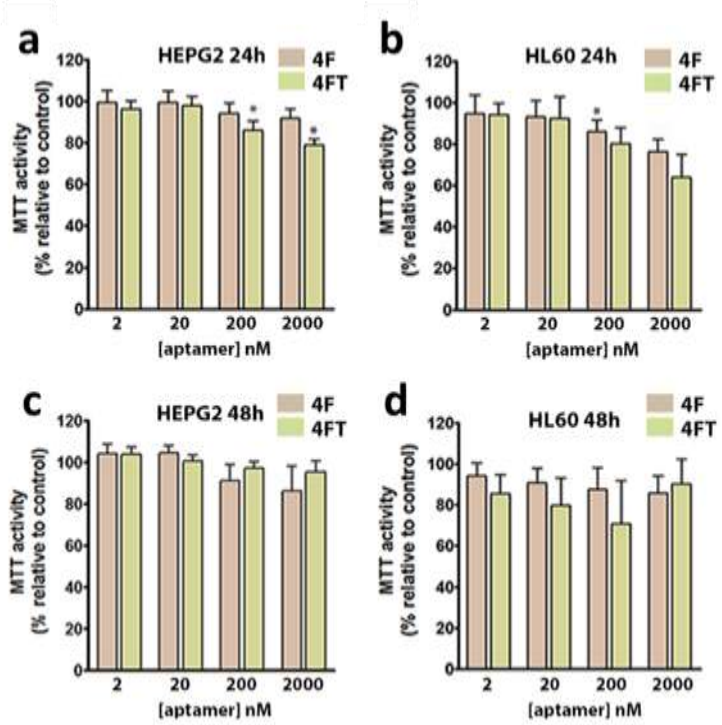

Figure S3. In vitro toxicity assays of ApTLR#4F and ApTLR#4FT. (a, b) Effect of the incubation of cell lines HEPG2 (**a**) and HL60 (**b**) with ApTLR#4F and ApTLR#4FT (2-2000 nM) on cell viability at 24 hours. At concentrations within the biological activity range (20 nM) aptamers showed no toxic effect on cell viability. At higher concentrations (200-2000 nM) a mild effect (~20% max) was observed. (**c, d**) Effect of the incubation of cell lines HEPG2 (**c**) and HL60 (**d**) with ApTLR#4F and ApTLR#4FT (2-2000 nM) on cell viability at 48 hours. Effects observed at 24 hours were no longer detectable at 48 hours. Data represent mean ± SEM, n=4, Student t-test (a and b) (*) $p<0.05$ vs control (100%).

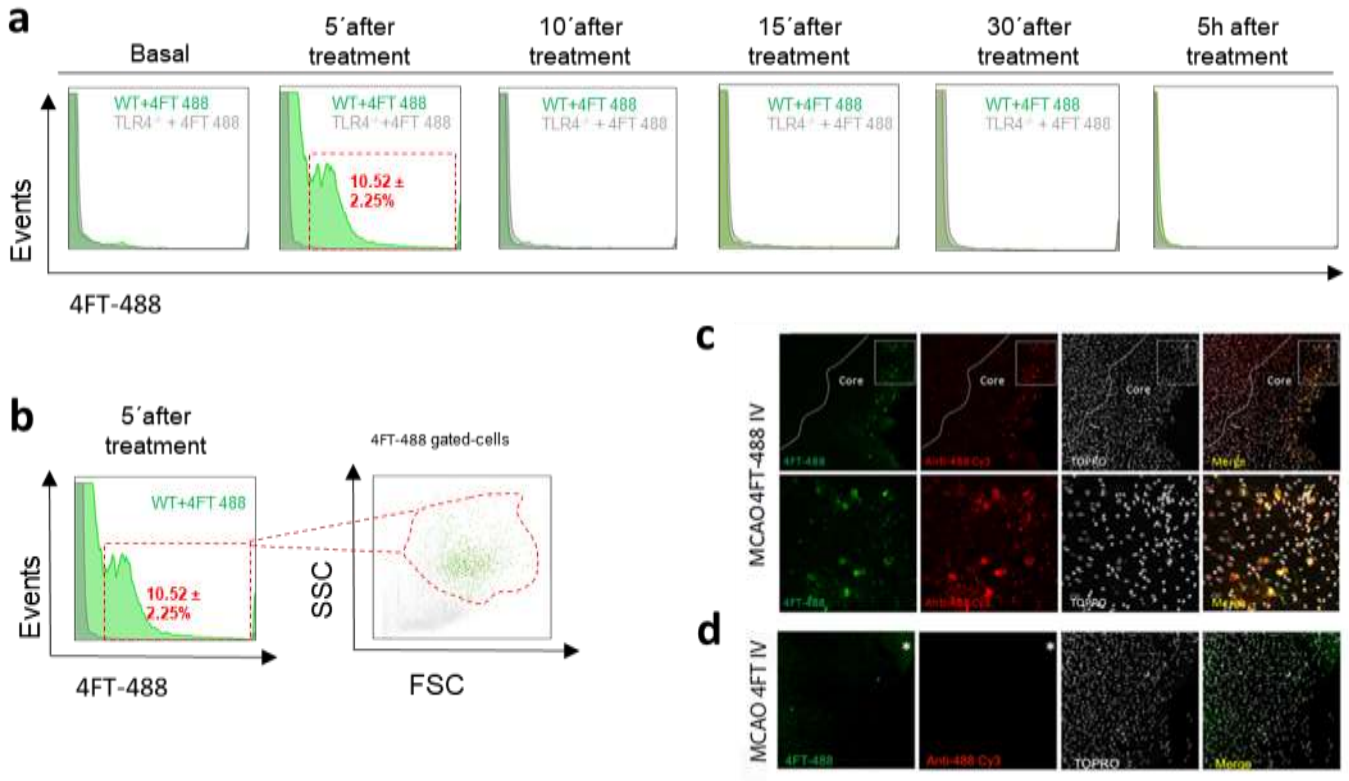

Figure S4. (**a**) Flow cytometric peripheral analysis of Alexa Fluor 488-labelled ApTLR#4FT (4FT-488; 1nmol) in WT and TLR4KO mice. (**b**) Alexa Fluor 488-labelled ApTLR#4FT in the granulocyte region at 5 minutes after aptamer administration in WT mice. (**c**) Distribution of Alexa Fluor 488-labelled ApTLR#4FT within the brain infarcted region 24 hours after intravenous injection. Pattern of distribution of the aptamer within the ischemic core (green), confirmed by probing with an anti-Alexa-488 antibody conjugated with Cy3 (c; red). Unconjugated ApTLR#4FT was used as negative control (**d**).

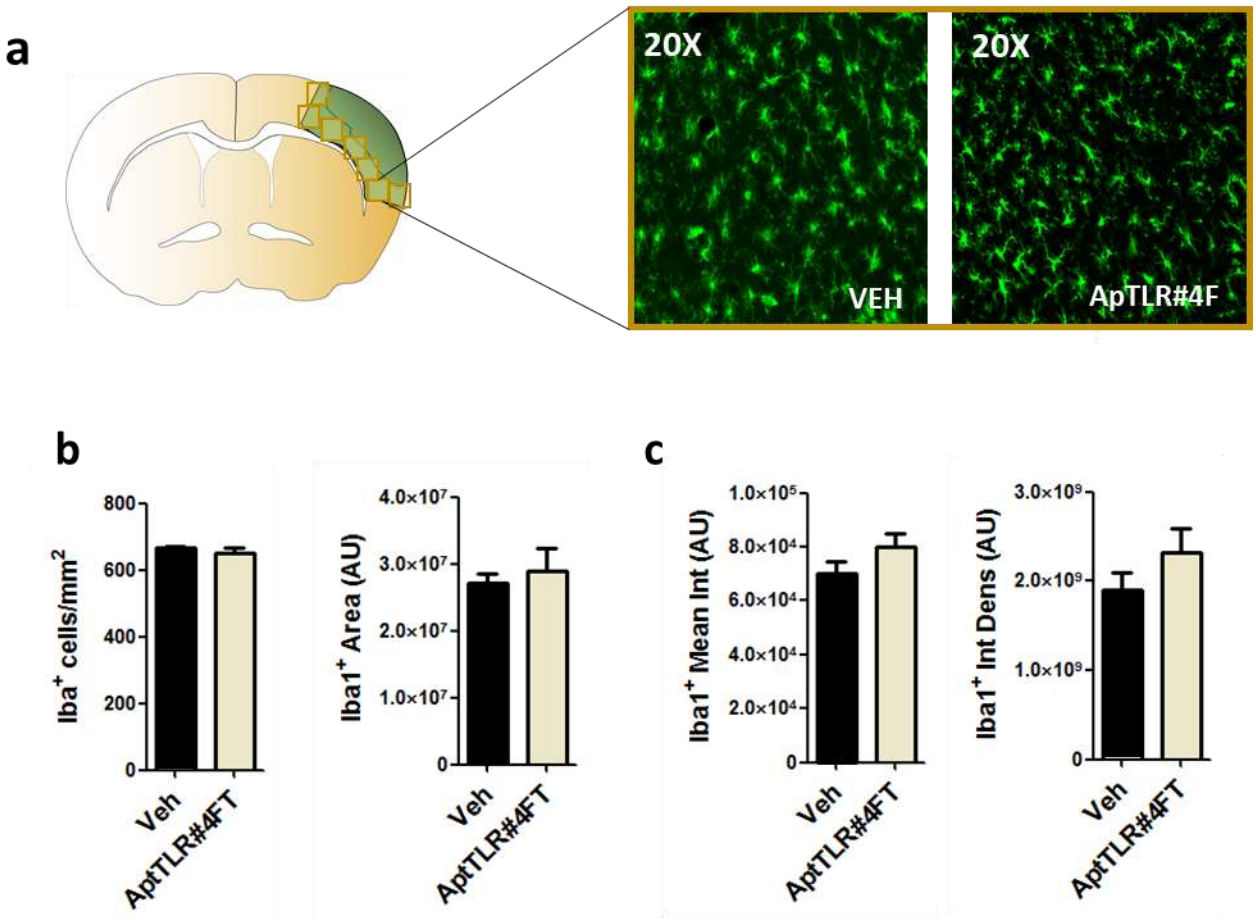

Figure S5. Effects of ApTLR#4FT on microglial activation after pMCAO. (**a**) Representative images of Iba1 cells in ipsilateral peri-infarct hemispheres in vehicle or ApTLR#4FT treated animals. (**b**) Quantification of microglial numbers or area (Iba1+ cells) and (**c**) Iba1+ mean intensity (Mean Int) and integrated density (Int Dens) of peri-infarct region in pMCAO mice treated either with vehicle or ApTLR#4FT. n=4 in each group. Data are expressed as mean±SEM.

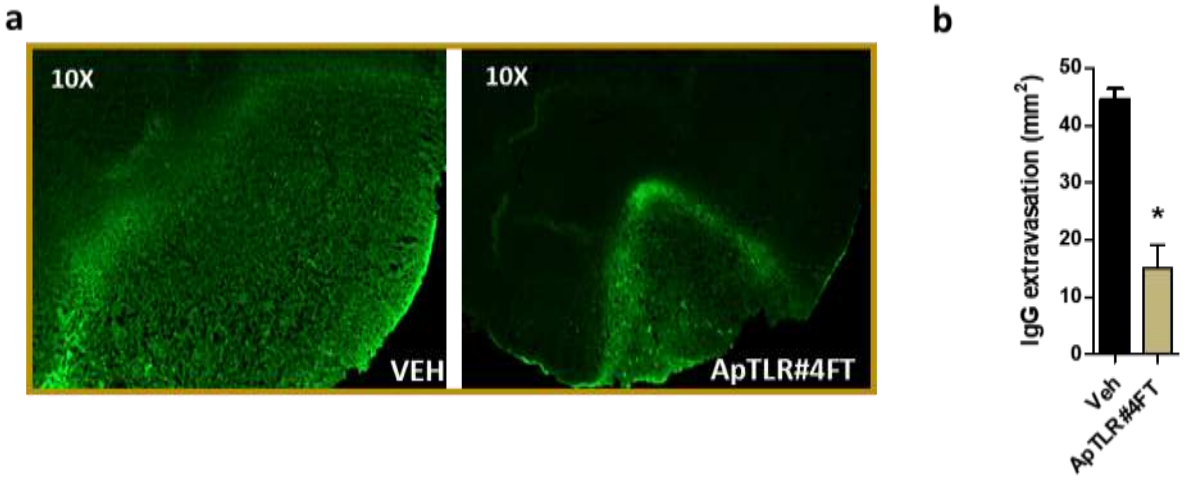

Figure S6. Effect of ApTLR#4FT on IgG extravasation after pMCAO. (**a**) Representative images of IgG extravasation in ipsilateral hemispheres in vehicle or ApTLR#4FT treated animals. (**b**) IgG extravasation area (mm2) in pMCAO mice treated either with vehicle or ApTLR#4FT. Data are obtained 24h after ischemia and are mean ± SEM; n=4 in each group; Student t-test (*$p<0.05$ vs Veh).

Aptamer	Sequence	nt	%A	%T	%G	%C
ApTLR#1R	gttgctcgtatttagggccaccggcacgggacaaggcgcgggacggcgtagatcaggtcgacaccagtcttcatccgc	78	19	18	33	29
ApTLR#1F	gcggatgaagactggtgtcgacctgatctacgccgtcccgcgccttgtcccgtgccggtggccctaaatacgagcaac	78	17	19	30	34
ApTLR#2R	gttgctcgtatttagggcacacacgcacgaagaccttggctgcccgttgtacaccagtcttcatccgc	68	19	25	24	32
ApTLR#2F	gcggatgaagactggtgtacaacgggcagccaaggtcttcgtgcgtgtgtgccctaaatacgagcaac	68	25	19	32	24
ApTLR#3R	gttgctcgtatttagggcaccgaggtcaccgaacttggtgtgcacagttgttggcgcgacaccagtcttcatccgc	76	17	26	29	28
ApTLR#3F	gcggatgaagactggtgtcgcgccaacaactgtgcacaccaagttcggtgacctcggtgccctaaatacgagcaac	76	26	17	28	29
ApTLR#4R	gttgctcgtatttagggccaaccgagtacatgctaacgcggcgatatggtttattggcacaccagtcttcatccgc	76	21	28	25	26
ApTLR#4F	gcggatgaagactggtgtgccaataaaccatatcgccgcgttagcatgtactcggttggccctaaatacgagcaac	76	27	21	26	25
ApTLR#5R	gttgctcgtatttagggccacatatgtgcacatcacaatccgcagagctgcacctacgacaccagtcttcatccgc	76	23	24	20	33
ApTLR#5F	gcggatgaagactggtgtcgtaggtgcagctctgcggattgtgatgtgcacatatgtggccctaaatacgagcaac	76	23	24	33	20
ApTLR#6R	gttgctcgtatttagggccaaggaaaaccccctggtcactggtactaatccgatccgtacaccagtcttcatccgc	76	22	25	21	32
ApTLR#6F	gcggatgaagactggtgtacggatcggattagtaccagtgaccaggggttttccttggccctaaatacgagcaac	76	24	22	32	21
ApTLR#7R	gttgctcgtatttagggcgggtcaccacggaagagtgtagatacatagatacagtccgacaccagtcttcatccgc	76	24	24	26	25
ApTLR#7F	gcggatgaagactggtgtcggactgtatctatgtatctacactcttccgtggtgacccgccctaaatacgagcaac	76	23	25	25	26

Table S1. Sequences of the aptamers obtained after 6 rounds of SELEX. The number and percentage of each nucleotide are shown.

	Basal	Ischemia	Reperfusion
VEHICLE	119.23 ± 40.30	11.48 ± 4.47	109.28 ± 52.36
4FT	117.92 ± 45.37	14.52 ± 4.75	106.93 ± 36.84

Table S2. CBF values (mean and SD) for all treatment groups in rat tMCAO.

SUPPLEMENTARY MATERIALS AND METHODS

Protein-SELEX procedure

In the first round of the protein-SELEX procedure, 1 nmol of RND40 population, denatured at 90°C for 10 min and then cooled on ice for 10 min, were mixed with 7 µg (100 pmol) of rhTLR4 in 200 µL of SELEX buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM $MgCl_2$) and incubated at 37°C for 1 h (rounds 1-3) or 30 min (rounds 4-6) as above. The bound aptamer-hTLR4 complexes were purified using Ni-NTA superflow (Qiagen) and, after washing three times with SELEX buffer, the ssDNA-protein complexes were resuspended in 20 µL of distilled H_2O and amplified by 15 cycles of PCR using F3 and R3 primers in PCR buffer under the conditions of 1 µM/primer, 250 µM dNTPs, in a final volume of 100 µL as above. Contraselection steps were performed before the initial round and after rounds 3^{th} and 6^{th} of the SELEX procedure with Ni-NTA superflow resin.

In all the cases, preliminary PCR reactions were performed at different number of cycles in order to avoid artefacts produced when high amounts of template are used. PCR reactions at the end of the selection rounds showed increasing amounts of DNA product but they were not saturated which is consistent with recovery of increasing amounts of aptamers targeting rhTLR4 throughout the SELEX process. PCR product was ethanol-precipitated and quantified in a Nanodrop instrument. After each round of SELEX, isolated aptamers were denatured and then allowed to refold before starting the second and all subsequent rounds of SELEX.

Aptamer cloning, sequencing and secondary structure prediction

After 6 rounds, the selected aptamer populations were amplified by using 1U Taq DNA polymerase (Biotools, Spain) in 50 µL of reaction also containing 1xPCR buffer, 125 mM dNTPs, 1 µM F3 primer and 1 µM R3 primer. The dsDNA product with ‘A’-overhangs was cloned into pGEM-T Easy-cloning vector (Promega, USA) following manufacturer’s instructions. Individual

clones were sequenced using T7 (5´-TAATACGACTCACTATAGGG-3´) and Sp6 (5´-ATTTAGGTGACACTATAGAA-3´) primers. Selected ssDNA molecules were subjected to secondary structure prediction using the mFold version 3.5 software (http://mfold.rna.albany.edu/) 30 at 37°C in 150 mM NaCl and 1 mM $MgCl_2$ and QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS) a web-based server for predicting G-quadruplex in nucleotide sequence 39.

Aptamer resistance to nucleases

Three hundred ng of aptamer were incubated with 1 unit of λ-exonuclease or DNAse I (Fermentas) in a 10 µL reaction for 5 min, 15 min, 30 min, 1 h, 2 h and 4 h at 37°C. In another set of experiments, 5 µL of buffer containing the same amount of aptamer were mixed with 10 µL of human plasma and incubated for 0 h. 0.5 h, 1 h, 2 h, 4 h, 20 h and 30 h at 37°C. Afterwards, samples were resolved on denaturing polyacrylamide gels prepared with final concentrations of 3.5 M urea, 10% acrylamide, and 1× TBE buffer. Polyacrylamide was prepared from a commercially obtained stock solution of 40% acrylamide in water with a 19:1 ratio of acrylamide:bis-acrylamide (Pronadisa, Spain). The samples were loading in gel with 3M urea final loading buffer. Bands were visualized by SYBR® Green I (Life Technologies, USA) post electrophoresis stain and quantified using ImageQuant^{TL} (GE Healthcare) software.

Cell viability assays

Toxicity of the ApTLR#4F and ApTLR#4FT was analysed with cell viability (MTT) and necrotic cell death (LDH) assays using 2 cell lines (Hep-G2 and HL-60) and different concentrations of the aptamers (2-2000 nM) at 24 and 48h. In both types of assays, cells (10^4 cells/well) were plated onto 96-well plates. Twenty-four hours later, the culture medium was replaced by fresh medium

containing increasing concentrations (2 nM-2 μM) of the aptamers and cultured at 37°C in 5% CO_2 for 24 h or 48 h. In cell viability assays, after incubation with aptamers, 25 μL of MTT (5 mg/mL) were added to each well and plates were incubated at 37°C for 4 h. Next, crystals were dissolved with DMSO and absorbance was read at 540 nm on a microplate reader (SpectraFluor, TECAN). The percentages of viable cells were then determined by reduction of MTT relative controls. All data shown are the means of six independent experiments.

Blood cells characterization by flow cytometry

Peripheral blood from Alexa Fluor 488-labelled ApTLR#4FT in WT and TLR4KO treated-animals was incubated for 10 minutes with a standard ammonium chloride lysing solution. Samples were washed twice with phosphate-albumin buffer (PAB; 0.0455% sodium azide, and 0.1% bovine serum albumin) and resuspended in PAB and mouse Fc Block (1:500; BD Pharmingen). Cells were washed and resuspended in 300μl of FACS Flow (BD Pharmingen). 15000 events of total gated cells were acquired using a FACSCalibur flow cytometer with CellQuest software (BD Pharmingen, San Jose, CA). Granulocytes were identified by forward and side scatter analysis.

Immunofluorescence and confocal microscopy

Free-floating coronal brain slices (30 mm) were processed as we described previously in Materials and Methods. In brief, brain sections were blocked with 5% goat serum and incubated with rabbit polyclonal anti-IBA1 receptor (WAKO Pure Chemical Industries Ltd. #019-19741), 24h at 4Cº, followed by a rabbit secondary antibody Alexa 488 (Invitrogen A-11008) (1h, RT).

All immunofluorescence images were obtained in a blinded manner from seven correlative slices of each brain. With the ImageJ v. 1.44l software (NIH, Bethesda, MD, USA), each image was converted into a binary image and the Integrated Density (Int Dens) was calculated. The Int Dens is a calculus

of the mean stained area times the intensity of stain in each pixel in the area, and indicates the total amount of staining material in that area.

For microglial quantification (Iba^+ cells), immunofluorescence images were taken from five correlative sections beginning in 1.70 mm from the bregma (until 0.02 mm). The images were taken at 20× and spaced 400 μm from each other of the upper and lower part of the peri-infarcted tissue. The entire top of the cortex was traced and 800 μm below the stroke, using as boundaries of the corpus callosum and the end of the cortex, by analyzing a total of about 5 images per hemisphere ipsilateral and section. The number of Iba+ cells was quantified on digitalized confocal images captured from six serial 40-μm sections spaced 0.32 mm apart (two fields of view per section; Zeiss LSM 710) in ipsi- and contralateral cortices. Three-dimensional localization of protein marker was validated using confocal z-stacks.

Assessment of blood-brain barrier permeability

IgG extravasation was determined as an indicator of BBB opening. The quantification was achieved using an Eclipse E600 (Nikon Corporation, Japan) microscope, a 10X objective and the software Stereo Investigator (Visiopharm, Denmark). The summation of the positive area of eight sections in the ipsilateral cortex was further estimated, from bregma 1.6 to -2.4 (one every 400 μm), using the application of the Cavalieri's principle.