CHAPITRE 5 : Ciblage thérapeutique au sein des cellules endothéliales du cerveau via l'utilisation d'immunoliposomes PEGuylées ciblant le récepteur de la transferrine

Résumé

Les cellules endothéliales des capillaires du cerveau (CECCs) forment le cœur de la barrière hémato-encéphalique (BHE) en expansion dans tout le cerveau. Les CECCs sont impliqués de manière dynamique dans le contrôle de l'homéostasie du SNC et représentent des cibles thérapeutiques potentielles pour le traitement des maladies reliées au cerveau. Causer par leur grande proximité avec les neurones et les astrocytes, les CECCs procurent aussi une opportunité unique pour distribuer globalement un agent thérapeutique dans tout le cerveau. Dans cette étude, nous avons utilisé une formulation PEGuylée d'immunoliposomes conjuguée avec le Ri7, un anticorps ciblant le récepteur de la transferrine (RTf), pour transporter des acides nucléiques (ARN double brin ou ADN plasmiques) marqués en fluorescence dans les CECCs *in vitro* et *in vivo*. Les expériences menées sur des cellules en culture de type N2A et bEnd5 ont montré que l'accumulation cellulaire des immunoliposomes était spécifique au RTf. L'accumulation cellulaire a été confirmée par la colocalisation avec les marques d'endosomes précoces (EEA-1) et RTf. Les expériences *in vivo* ont montré que l'encapsulation d'acides nucléiques dans les immunoliposomes conjugués avec le Ri7 menait à une accumulation dans les CECCs après une injection par intraveineuse chez la souris. Nos résultats suggèrent que les acides nucléiques peuvent être acheminés dans les CECCs en utilisant une formulation d'immunoliposomes ciblant le RTf, ouvrant ainsi la porte à l'utilisation potentielle des CECCS comme cible de traitement pour les pathologies du SNC.

Brain endothelial cells drug delivery PEGylated Ri7-targeted immunoliposomes

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Abstract

Brain capillary endothelial cells (BCECs) form the core of the blood-brain barrier (BBB) expanding throughout the brain. BCECs are dynamically involved in CNS homoeostasis and harbor potential drug targets to treat brain diseases. Due to their close proximity to neurons and astrocytes, BCECs also provide unique opportunities for widespread distribution of drugs into the CNS. In this study, we have utilized PEG-stabilized immunoliposomes (PSIL) conjugated with Ri7, an antibody targeting the transferrin receptor (TfR) to deliver fluorescently tagged nucleic acids (double-stranded RNA or pDNA) into BCEC *in vitro* and *in vivo*. Experiments conducted in cultured N2A and bEnd5 cells showed that cellular uptake was specific to the TfR. Intracellular accumulation was confirmed by colocalization with an early endosome (EEA-1) and TfR marker. Experiments in mice showed that encapsulation of nucleic acids into Ri7-PSIL led to their distribution into BCEC after a single intravenous injection. Our results show that nucleic acids can be ferried into BCECs using PSILs targeting the TfR, thereby opening the door to the potential utilization of BCECs has targetable cells for CNS therapeutics.

Background

Brain capillary endothelial cells (BCECs) play a key role in brain homoeostasis, controlling the access to cerebral parenchyma for all blood-borne molecules. Due to their structural role the brain-blood barrier (BBB), BCECs are traditionally seen as the main obstacle to the development of treatments for brain pathologies (Scherrmann 2002, Freskgård et al. 2014, Pardridge 2015b). Indeed, the vast majority of small molecules and virtually all large biopharmaceuticals cannot cross the BCECs layer of the BBB (Pardridge 2007b, Zlokovic 2008b, Pardridge 2012). However, as much as BCECs prevents drug from entering the cerebral parenchyma, they could also act as a launching platform to reach brain cells (Paris-Robidas et al. 2011, Alata et al. 2014, Assmann et al. 2016, Körbelin et al. 2016, Paris-Robidas et al. 2016). Indeed, BCECs express a multitude of specific transport systems involved in endocytosis and transcytosis (Broadwell et al. 1996, Descamps et al. 1996, Carpentier et al. 1999, Fillebeen et al. 1999) that can be targeted for drug delivery (Watts et al. 2013, Pardridge 2015b, Pardridge 2015a, Freskgård et al. 2017). Moreover, many studies have highlighted the crucial role played by BCECs in brain disorders such as neurodegenerative diseases (Zlokovic 2002, Bell et al. 2009, Weiss et al. 2009a, Rosenberg 2012). Therefore, directly targeting BCECs could be an interesting approach to treat brain disorders (Paris-Robidas et al. 2011, Assmann et al. 2016, Körbelin et al. 2016).

There is currently no curative treatment for neurodegenerative disease. Gene therapy is an attractive strategy for the development of disease modifying treatment (y Ribotta 2001b). However, systemic instability of naked nucleic acids limit their bioavailability in target tissues and can barely accumulate in the brain (Leung et al. 2014, Lorenzer et al. 2015). Considering their high transfection efficiency, viral vectors have been widely used over the past decades (Collins et al. 2015, Choudhury et al. 2017). However, due to safety concern, lipid-based nanoparticles have been developed to offer a safer alternative to viral transfection (Huang et al. 2009, Patidar et al. 2010). Liposomes can be formulated to deliver their content in certain subcellular components (Thekkedath et al. 2013, Biswas et al. 2014) and they can be targeted to specific tissues after systemic administration by chemical coupling to monoclonal antibody (mAb) (Meissner et al. 2015, Broekgaarden et al. 2016, Greineder et al. 2016). The transferrin receptor (TfR) has been widely study owing to its enrichment in BCECs and ability to trigger endocytosis (Harding et al. 1984, Roberts et al. 1993, Kissel et al. 1998, Enerson et

al. 2006, Moos et al. 2007, Johnsen et al. 2016). Preclinical studies have shown some success for TfR-targeted delivery into BCECs, particularly when using high affinity (Shin et al. 1995, Yu et al. 2011, Zhou et al. 2011c, Yu et al. 2014). More specifically, recent publications have highlighted the clone Ri7, a mAb raised against the murine TfR as a potential vector to directly trigger endocytosis into BCECs (Paris-Robidas et al. 2011, Alata et al. 2014, Paris-Robidas et al. 2016). Our group has developed a PEG-stabilized immunoliposomes (PSILs) formulation featuring high DNA encapsulation rate and TfR-specific transfection efficacy *in vitro* (Rivest et al. 2007). This formulation with a half-life of 24 h is also adapted for *in vivo* utilization (Rivest et al. 2007). Moreover, it is composed of natural and biodegradable phospholipids known for their a low level of immunogenicity and cellular toxicity (Orive et al. 2004, Goyal et al. 2005, Schnyder et al. 2005, Rivest et al. 2007, Mallick et al. 2014).

In order to study the capacity of liposomes to ferry nucleic acids into brain microvasculature, we conjugated the mAb Ri7 to PSILs in which either fluorescent pDNA or a fluorescent double-stranded (ds) RNA analog (siGLO Red) were encapsulated. Cellular uptake was assessed *in vitro* whereas brain distribution was investigated *in vivo*. Experiments on cultured cells showed that the uptake of Ri7-conjugated PSILs was mediated by the TfR and that the nanocomplex was incorporated into endosomal compartments such as early endosomes and lysosomes. Intravenous administration of Ri7-PSILs led to detection of fluorescently tagged siGLO Red or pDNA throughout the microvasculature of the mouse brain.

Materials and methods

mAbs radiolabeling

One mCi of N-succinimidyl-[2,3-^{3}H] propionate ([^{3}H]-NSP, 101 Ci/mmol) was evaporated under a nitrogen stream in a borosilicate tube and incubated with 500 mg of mAbs into 100 ml of a buffer containing 0.1 M sodium borate/1.5 M NaCl (pH 8.0) for 1 h at room temperature, followed by a 2 h incubation at 4°C. Radiolabeled mAbs were separated using a G-25 Sephadex gel filtration column (GE-Healthcare-Amersham, Baie d'Urfé, QC, Canada) pretreated with 10% (w/v) BSA (Sigma-Aldrich, Oakville, ON, Canada), and specific activity was computed using disintegrations per minute (dpm) counts and protein content in eluted fractions.

DNA labeling

DNA was labeled using the ULYSIS nucleic acid labeling kit (Alexa Fluor 546, AF546) following the manufacturer's instructions (ThermoFisher/Life Technologies). Briefly, two generic plasmids were digested with restriction enzymes to generate equimolar linear DNA fragments of size < 1.1 Kb (1097, 1069, 1057, 1008, 896, 179 and 1008, 725, 610, 472, 467, 370, 105 bp) that were purified by ethanol precipitation. Then, 200 µg were resuspended in 1200 µl of labeling buffer, denatured at 95°C and kept on ice while 3 vials of ULS labeling reagent were prepared and pooled in 300 µl 50% dimethylformamide. Denatured DNA was then incubated with labeling reagent at 80°C for 45 min and unbound fluorophores as well as solvant were washed away with Montage columns (Millipore, Etobicoke, ON, Canada). Degree of labeling was calculated at one molecule of AF546 for each 38 bp of DNA and 200 µg in 200 µl H2O were added to the lipids. Water was used for encapsulation instead of ethanol.

PEGylated stealth immunoliposomes (PSILs) preparation

Briefly, POPC(1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine) (Northern Lipids Inc., Vancouver, BC, Canada) (18.6 mmol), DDAB (Didodecyldimethylammonium bromide) (Sigma-Aldrich) (0.6 mmol), DSPE-PEG$_{2000}$ (distearoylphosphatidylethanolamine)-PEG$_{2000}$ (2,000 Da polyethylene glycol) (Northern Lipids Inc.) (0.6 mmol) and the linker lipid DSPE-PEG$_{2000}$-maleimide (0.2 mmol)

(Nektar Therapeutics, Huntsville, AL) were dissolved in chloroform, followed by evaporation under a nitrogen stream and constant agitation in order to produce a thin lipid film, which was allowed to dry during 30 min. The dried lipid film was dispersed in 0.2 ml 0.05 M Tris-HCl (pH 7.0), vortexed for 2 min, followed by a 10 min incubation, 10 min of bath sonication and a second 1-min vortex agitation. Fluorescent dsRNA analog siGLO Red (Dharmacon, Ottawa, ON, Canada) (40 nmoles), or fluorescent DNA (200 µg) were added to the lipids. Then, 0.6 ml of a 67 % (v/v) ethanol solution in 0.05 M Tris-HCl buffer (pH 7.0) was added drop by drop during 30 sec under minimal agitation. Large vesicles were converted into liposomes under 100 nm by extrusion using a hand-held LiposoFasti-Basic extruder (Avestin, Ottawa, ON, Canada) as described (Shi, Y Zhang, et al. 2001; Yun Zhang, Calon, et al. 2003). mAbs were conjugated to the freshly formed liposomes by adding three mg of unlabeled mAbs (RI7 or 2a3; BioXcell West Lebanon, NH) and 0.5 mCi of tritiated mAbs were thiolated with a 40:1 molar excess of freshly prepared 2-iminothiolane (Traut's reagent, Sigma-Aldrich) following a 1-h incubation at room temperature in 0.05 M sodium borate/0.1 mM EDTA (pH 8.5). Thiolated mAbs were transferred to a buffer containing 0.05 M HEPES/0.01 mM EDTA (pH 7.0) by centrifugation in a Vivaspin (Sartorius Stedim Biotech, Aubagne, France) filter unit (MWCO 30 kDa). This step was repeated twice and the final volume was reduced to ~200 µl. Afterwards, thiolated mAbs were added to the liposome preparation for an overnight room temperature incubation in a 2 ml glass bottle under inert nitrogen atmosphere. siGLO Red and fluorescent pDNA encapsulation and mAb conjugation efficiencies were determined by size exclusion chromatography (Figure 1). PSILs were eluted with 0.05 M HEPES (pH 7.0) at a rate of 1.0 ml/min in a 1.5 cm 20 cm pre-equilibrated Sepharose CL-4B column (GE-Healthcare-Amersham, Baie d'Urfé, QC, Canada). Fractions were quantified to determine their ^{3}H and fluorescent content by liquid scintillation and fluorescence quantification using a Synergy HT (Biotek, Winooski, VT). Eluted fractions containing PSILs were kept in glass vials under nitrogen atmosphere and stored at 4°C unless specified otherwise.

In vitro uptake and colocalization analyses

Murine neuroblastoma (N2A, #CCL-131, ATCC, Manassas, VA) and murine brain endothelioma (bEnd5, #96091930, HPACC, Salisbury, UK) cells were seeded in 96-well plates containing DMEM supplemented with 10% FBS at 4 x 10^5. Two days later, medium was removed and cells were incubated with DMEM-10% FBS containing Ri7-PSIL containing siGLO Red (500 nM) for 15, 45, 60

min, 4 and 24 h. Afterwards, cells were washed with ice-cold DMEM-FBS 10%, 1X D-PBS and fixed with 4% paraformaldehyde (PFA) for 15 min. For colocalization analyses, following the fixation, cells were permeabilized with D-PBS containing 0.5% Triton X-100 for 10 min and blocked with a 3% bovine serum albumin (BSA), 3% normal horse serum (NHS) and 0.05% Triton X-100 during 45 min. Cells were then incubated overnight at 4°C with primary antibodies in a D-PBS solution with 1% BSA, NHS and Triton X-100: rabbit anti-EEA-1 (1:200, NEB Cell Signaling, ON, Canada), rabbit anti-TfR ab84036 (1:200, Abcam, ON, Canada) and rabbit anti-LAMP-1 (1:2000, Sigma-Aldrich). Following the incubation with primary antibodies, cells were exposed to Alexa-Fluor™-conjugated donkey anti-rabbit (ThermoFisher Scientific), cell nuclei were counterstained with DAPI (Sigma-Aldrich) and IBIDI mounting media was added. Images were acquired with an EVOS™ FL auto imaging system (ThermoFisher Scientific).

In vitro competition studies

N2A cells were seeded in 96-well plates containing DMEM supplemented with 10% FBS at 5 x 10^5. The next day, medium was removed and cells were preincubated with Ri7 or control IgG (2a3) (0, 50 or 200 nM) for 30 min. Then, Ri7-PSILs were added to a final siGLO Red concentration of 250 nM and incubation was resumed for 15 min. Incubation was terminated by sequential washes with ice-cold DMEM-FBS 10% and D-PBS 1X. Afterward, cells were fixed with 4% PFA, cell nuclei were counterstained with DAPI (Sigma-Aldrich) and IBIDI mounting media was added. Images were acquired with an EVOS™ FL auto imaging system.

Animals

Adult Tie2GFP mice (Jackson Laboratory, Bar Harbor, ME) weighting 20-30 g were used. They had ad libitum access to food and water. All procedures were performed in accordance with the Canadian Council on Animal Care standards and were approved by the Animal Ethics Committee of the *Centre Hospitalier de l'Université Laval* (CHUL).

Tissue preparation for post-mortem analysis

For microscopy experiments mice were systemically injected with Ri7- or 2a3-PSILs containing 20 µg (1.33 mg/kg) of siGLO Red, pDNA546 and sacrificed 1 h post injection by terminal transcardiac

perfusion under deep anesthesia with ketamine/xylazine. Animals were perfused with 25 ml of cold 1X PBS followed by 50 ml of 4% PFA, pH 7.4. Brains were rapidly dissected, post-fixed with 4% PFA for 4 h and cut in coronal sections of 25 µm with a microtome (Leica Microsystems, Richmond Hill, ON, Canada). For TfR levels quantification animals were injected twice a day for two days and sacrificed by transcardiac perfusion with 1X PBS containing protease inhibitors (SigmaFast protease inhibitor tablets, Sigma-Aldrich) 24 h following the last injection. Brain and liver were collected and weighed.

Brain microvessels isolation

Mouse brain microvessels were isolated by density-gradient centrifugation using the capillary depletion technique as previously described (Alata et al. 2015; Do et al. 2014). Briefly, brains were collected and transferred into ice-cold 1X PBS. Cerebellum, meninges and brain stem were removed. Afterward, brains were gently homogenized in ice-cold DMEM containing 10% FBS using a Potter homogenizer. The homogenates were centrifuged at 500 xg for 10 minutes at 4°C, the supernatant was excluded and the pellets were homogenized in 5 ml of ice-cold DMEM containing 25% BSA and centrifuged at 1,500 xg for 20 minutes at 4°C. The resulting pellets were suspended in 1 ml of ice-cold DMEM with 10% FBS and the homogenate was filtered through a 60-µm filter. The filtrates were centrifuged at 12,000 xg for 45 minutes at 4°C. The pellets containing the microvessels were washed in ice-cold 1X PBS and centrifuged again at 12,000 xg for 20 minutes at 4°C. The supernatants were discarded and the pellets were stored at - 80°C until processed for western blotting analysis.

Proteins extraction

Proteins were extracted from tissues as previoulsy described (St-Amour et al. 2014). Briefly, the pellet containing the microvessels was weighed and total proteins were extracted by homogenization in four volumes of lysis buffer (150 mmol/L NaCl, 10mmol/L NaH_2PO_4, 1% Triton X-100, 0.5% SDS, and 0.5% deoxycholate, pH 7.4) containing Complete protease inhibitors (Roche, Indianapolis, IN, USA) and 10 mg/ml pepstatin A (Sigma-Aldrich). The obtained suspension was sonicated briefly (3×10 seconds) and centrifuged at 100,000 xg for 20 minutes at 4°C. Supernatant was stored at −80°C until western blotting analysis.

Western Blotting

Equal amounts of proteins per sample were added to Laemmli's loading buffer, heated to 95°C for 5 minutes before loading (20 µg protein per lane), and subjected to sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis. Proteins were electroblotted onto PVDF membranes (Immobilon, Millipore, MA, USA) before blocking in 5% non-fat dry milk, 0.5% BSA, and 0.1% Tween-20 in 1X PBS for 1hour at room temperature. The membranes were washed three times for 10 minutes in 1X PBS containing 0.1% Tween-20. Then, membranes were incubated overnight at 4°C with rabbit anti-TfR primary antibody (1:1000, Abcam) diluted in 1X PBS containing 0.1% Tween-20, 5% non-fat dry milk, and 0.5% BSA. The next day, membranes were washed three times for 10 minutes in 1X PBS containing 0.1% Tween-20 and then incubated for 1 hour at room temperature with goat anti-rabbit horseradish peroxidase-labeled (1:60 000, Jackson, West Grove, PA, USA) secondary antibody diluted in 1X PBS containing 0.1% Tween-20 and 1% BSA. The membranes were again washed three times for 10 minutes in 1X PBS containing 0.1% Tween-20 and probed with chemiluminescence reagents (Luminata, ThermoFisher Scientific). Immunoblots were analyzed with a KODAK Imaging Station 4000MM Digital Imaging System (Molecular Imaging Software version 4.0.5f7, Carestream Health, Rochester, NY, USA).

Immunofluorescence

Washing steps were performed using 1X PBS, pH 7.4, between every step of the immunofluorescence protocol. Coronal brain sections were blocked for 1 h with a 1X PBS solution containing 5% NHS (ThermoFisher Scientific) and 0.2% Triton X-100. Afterwards, sections were incubated overnight at 4°C with primary antibodies in the blocking solution: mouse anti-neuronal nuclei (1:1000, NeuN, Chemicon/Milipore, Temecula, CA) and mouse anti-GFAP (1:1000, Sigma-Aldrich). Following incubation with primary antibodies, slices were exposed to Alexa Fluor conjugated donkey anti-mouse and anti-rabbit secondary antibodies (1:1000, ThermoFisher Scientific). Finally, slides were then coverslipped with ProLong® Diamond antifade media (ThermoFisher Scientific).

Results

Uptake studies of Ri7-PSILs were performed on mouse N2A neuroblastoma cells and bEnd5 murine endothelial cells, which were incubated with Ri7- or 2a3-PSIL containing a fluorescent dsRNA analog (siGLO Red) for 15, 45, 60 min, 4 and 24 h (Figure 34). Uptake of the Ri7-PSIL complex was observed within 15 min and increasing over a 24 h time period, while no signal was visible in cells incubated with 2a3-PSIL (Figure 34). Pretreatment with 50 or 200 nm of free Ri7 blocked the accumulation of Ri7-PSIL in N2A to a level similar to the control nanoparticle (Figure 35A-D). In comparison, pretreatment with 2a3 mAb did not alter the uptake of Ri7-PSIL (Figure 35E). Finally, colocalization analyses showed a robust colocalization between TfR and Ri7-PSIL (Figure 35F). These results confirm the ability of Ri7-PSIL to ferry nucleic acids into cultured N2A or bEnd5 cells, via the TfR.

To investigate the cellular fate of Ri7-PSILs, we incubated N2A cells with the anti-TfR nanocomplex containing siGLO Red for 15 min, 1 or 4 h and performed colocalization experiments with intracellular markers by immunofluorescence. The results of the immunofluorescence with EEA-1 showed that after 1 and 4 h of incubation on N2A cells, Ri7-PSIL had enter endosomal compartments (Figure 36). However, colocalization between Ri7-PSIL and EEA-1 was barely visible after 15 min, suggesting a slow internalization process. Moreover, the cellular distribution of the fluorescent signal suggests that most fluorescence from siGLO Red in Ri7-PSIL was still located at the cellular membrane after 15 min (Figure 36).

To access whether Ri7-PSIL were directed toward lysosomal compartments, we performed colocalization experiments with N2A cells incubated with Ri7-PSIL for 15 min, 1 or 4 h and lysosomal marker LAMP-1 (Figure 37). Colocalization analyses at 15 min showed that Ri7-PSIL were not rapidly directed toward the lysosome. Colocalization between siGLO signal and LAMP-1 immunolabeling was hardly visible after 15 min, but suggest that Ri7-PSIL slowly accumulated into lysosomal compartments after 1 and 4 h. However, our data suggest only a partial accumulation after a 4 h incubation on N2A cells (Figure 37C).

To assess the brain targeting capacity of Ri7-PSIL, Tie2GFP mice were injected with Ri7- or 2a3-PSIL containing fluorescent pDNA (Figure 38) or dsRNA siGLO Red (Figure 39). Animals were sacrificed 1 h after the systemic administration. Data in Figure 38 show fluorescent pDNA encapsulated in Ri7-PSIL was extensively distributed into the cerebral microvasculature, colocalizing with with GFP signal from the Tie2GFP endothelial cells (Figure 38A-C). No signal was detectable in the brain of mice injected with fluorescent pDNA complexed with PSIL conjugated with the control IgG 2a3 (Figure 38D). Further experiments were conducted with Ri7-PSIL containing a dsRNA fluorescent analog (siGLO Red) (Figure 39). Similarly, clear signal was observed in the GFP-labeled cerebral microvasculature in animal injected with siGLO-Ri7-PSIL formulation, but not when PSIL were conjugated with a control antibody. Overall, using fluorescent pDNA (Figure 38) and siGLO Red (Figure 39) presented data show that Ri7-PSIL delivered genetic material to BCECs in the mouse *in vivo*. In all experiments conducted, no signal from the nanocomplex targeting the TfR was visible outside the brain vasculature.

Previous study using anti-TfR antibodies suggested that lysosomal accumulation could lead to both *in vitro* and *in vivo* TfR degradation and thus impaired the uptake of anti-TfR mAbs administered chronically (Bien-Ly et al. 2014). To investigate if Ri7-PSIL could be used for repeated administration without altering TfR levels, Tie2GFP mice were injected twice a day for two days with Ri7-PSIL. Quantification by Western blot on capillary depletion fractions showed no significant difference on TfR levels between mice injected with Ri7-PSIL and control uninjected mice (Figure 40). Thus, our data suggest that even though Ri7-PSIL tends to accumulate toward lysosomal compartments *in vitro*, TfR levels remained unchanged *in vivo* after repeated injection of anti-TfR nanoparticles.

Discussion

In this present work, we characterized the cellular uptake and the brain distribution of TfR targeted PSIL. Using fluorescent dsRNA analog siGLO Red our results showed a TfR specific uptake in N2A cells. Moreover, *in vitro* colocalization highlighted the incorporation of Ri7-PSIL into the endocytic pathway. Moreover, *in vivo* experiments evidenced the accumulation in to BCECs of fluorescently tagged dsRNA or pDNA encapsulated into Ri7-PSIL formulations administered systemically.

N2A and bEnd5 cell lines express the TfR and thus represent suitable *in vitro* models to investigate targeting capacity and cellular uptake of nanoparticles targeting the TfR (Rivest et al. 2007; Bhattacharya et al. 2008). Previous study by our group showed the transfection capacity of TfR-targeted PSIL into N2A cells (Rivest et al. 2007). In the present study, *in vitro* experiments were performed to investigate the uptake mechanism of Ri7-PSILs. We showed that the cellular uptake of Ri7-PSIL is TfR-specific as it was reduced by pre-incubation of N2A cells with anti-TfR mAb. Moreover, immunolabeling of the TfR showed colocalization between Ri7-PSIL and TfR. This results is consistent with our previous work using fluorescent mAbs and confirmed that Ri7-PSIL retained similar targeting properties as the mAb alone (Alata et al. 2014, Paris-Robidas et al. 2016).

Our subsequent *in vitro* experiments provided evidence that Ri7-PSIL were endocytosed and integrated into endosomal compartments of N2A cells. Spatial colabeling between Ri7-PSIL signal and TfR or early endosomes marker suggest that Ri7-PSIL were internalized by clathrin-mediated endocytosis (Roberts et al. 1993). Nanoparticles internalized through a clathrin dependent mechanism are often trafficked towards the lysosomes (Bareford et al. 2007, Sakhrani et al. 2013, Hasanzadeh Kafshgari et al. 2015). Moreover, it had been shown that high affinity mAbs targeting the TfR can enhanced lysosomal accumulation (Bien-Ly et al. 2014). However, immunolabeling using lysosomal marker LAMP-1 showed only partial colocalization between lysosomes and Ri7-PSIL. Generally, therapeutic agents delivered to cell must completely avoid lysosomal degradation (Lorenzer et al. 2015, Tammam et al. 2016). However, treatment of diseases associated with lysosomal defects require accumulation of the

drug into the lysosome and Ri7-PSIL could be a potential nanodevice to treat such pathologies (Aronovich et al. 2015, Scarpa et al. 2015). Treating chronic diseases would also imply repeated injection of the nanoformulation. Therefore, maintaining constant TfR level on BCECs is crucial to ensure significant uptake of the therapeutic agent (Bien-Ly et al. 2014). Nevertheless, it was previously reported that systemic injection of high affinity mAbs targeting the TfR can enhance TfR lysosomal degradation both *in vitro* and *in vivo* (Bien-Ly et al. 2014), our results clearly showed that after multiple injections of Ri7-PSIL, BCECs TfR levels remained unchanged in capillaries isolated from the mouse. However, the injected dose used in the previous study by Bien-Ly et al. is rather high (50 mg/kg) and might explain the difference between our results (Bien-Ly et al. 2014). To assure the potential utilization of Ri7-PSIL for chronic administration, TfR levels should be measure *in vitro* after various incubation times and *in vivo* after a longer repeated administration or a higher dose of nanoparticles.

Since naked nucleic acids have poor systemic stability and cannot naturally cross the BBB (Lorenzer et al. 2015, Wang et al. 2015), we performed *in vivo* experiments in mice to investigate brain delivery of fluo-tagged oligonucleotides. Tie2GFP mice expressing the eGFP gene under the Tie2 promoter were used to readily identify microvessels (Motoike et al. 2000) and injected with various immunoliposome formulations containing fluorescent nucleic acids. Our microscopic analysis demonstrated that Ri7-PSIL can protect various kind of potential therapeutic drug for CNS pathologies from degradation and enhanced systemic stability as BCECs were labeled by the fluorescent signal contained in PSILs. Such specific BCEC targeting is consistent with our previous work showing that systemically administered Ri7 accumulate and are restricted into brain microvasculature (Paris-Robidas et al. 2011, Alata et al. 2014, Paris-Robidas et al. 2016). Moreover, our data are also in keeping with reports others publications using anti-TfR mAbs conjugated to liposomes, gold nanoparticles and FITC-labeled (Gosk et al. 2004, Manich et al. 2013, Cabezón et al. 2015).

Overall, our experiments highlight the potential utilization of PSIL for targeted drug delivery to treat endothelial dysfunction in CNS pathologies. In the last decade, implication of BCECs in brain disorders have gained interest. BCECs are now believed to play a crucial role in the development or establishment of neurological pathologies (Deane et al. 2004b, Bell et al. 2007,

Alata et al. 2015, Zhao et al. 2015). Therefore, targeting directly BCECs could lead to novel approaches to successfully treat neurological pathologies (Jiang et al. 2003, Hino et al. 2006, Kuwahara et al. 2011). Moreover, given the partial lysosomal accumulation, PSIL targeting the TfR could be a useful device to treat diseases associated with a lysosomal dysfunction. Lysosomal storage diseases and also certain neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease have a neurological lysosomal pathology (Zhang et al. 2009b, Vitner et al. 2010, Ortolano et al. 2014, Scarpa et al. 2015).

Conclusion

Enabling biopharmaceutical delivery to the CNS is one of the core challenges hindering the development of new neurotherapeutics. The present study reports evidence of the accumulation into BCECs of fluorescent nucleic acids encapsulated in systemically administered TfR-targeted PSIL. These observations underscore the potential of targeted PSIL to deliver large therapeutic molecules at the doorstep of brain cells. Such an approach could be utilized for the development of a new treatment for CNS disorders.

List of abbreviations

BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; BSA, bovine serum albumin; ds, double-stranded; EEA-1, early-endosome antigen-1; mAb, monoclonal antibody; NHS, normal horse serum; PFA, paraformaldehyde; PSIL, PEG-stabilized immunoliposome; PSL, PEG-stabilized liposome; TfR, transferrin receptor.

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the Canadian Council on Animal Care standards and were approved by the Animal Ethics Committee of the *Centre Hospitalier de l'Université Laval* (CHUL).

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare no competing interest

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Authors' contributions

FC and SPR designed research; SPR, PB, ML and VE performed research; SPR and FC analyzed data; SPR, VE and FC wrote the paper.

Figures

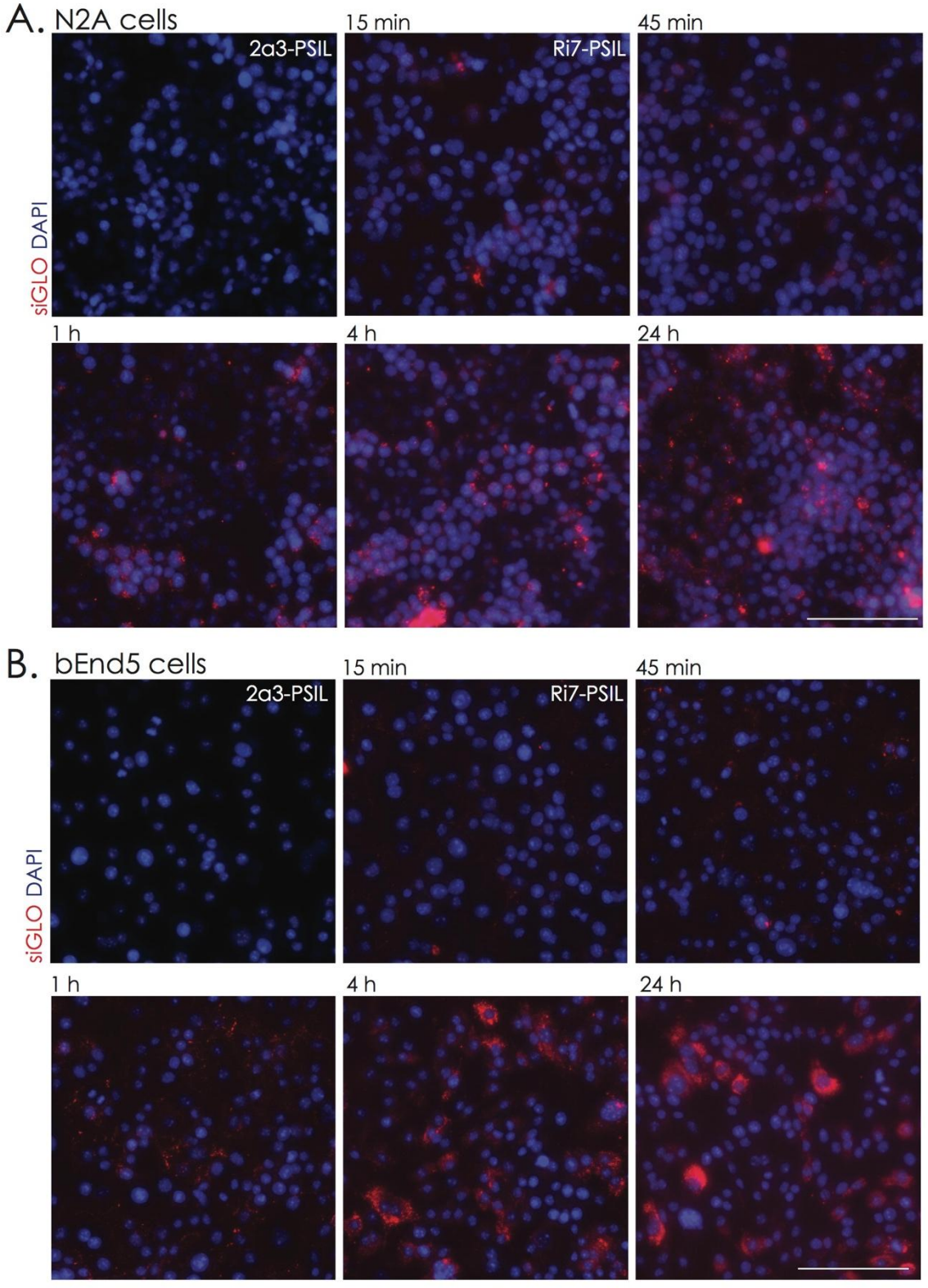
A. N2A cells
15 min
45 min
2a3-PSIL
Ri7-PSIL
siGLO DAPI
1 h
4 h
24 h
B. bEnd5 cells
15 min
45 min
2a3-PSIL
Ri7-PSIL
siGLO DAPI
1 h
4 h
24 h

Figure 40: Ri7-PSILs are internalized in N2A and bEnd5 cell lines.

(A) N2A and **(B)** bEnd5 cells were seeded in 96-well plates containing DMEM and 10% FBS. Two days later, they were exposed to Ri7- or 2a3-PSIL containing siGLO siRNA (red) (500 nM) for (15, 45, 60 min, 4 and 24 h) showing the accumulation over time in both cell lines. Cells were then washed with cold PBS, fixed with 4% paraformaldehyde and stained with DAPI (blue). Scale bar = 100 µm.

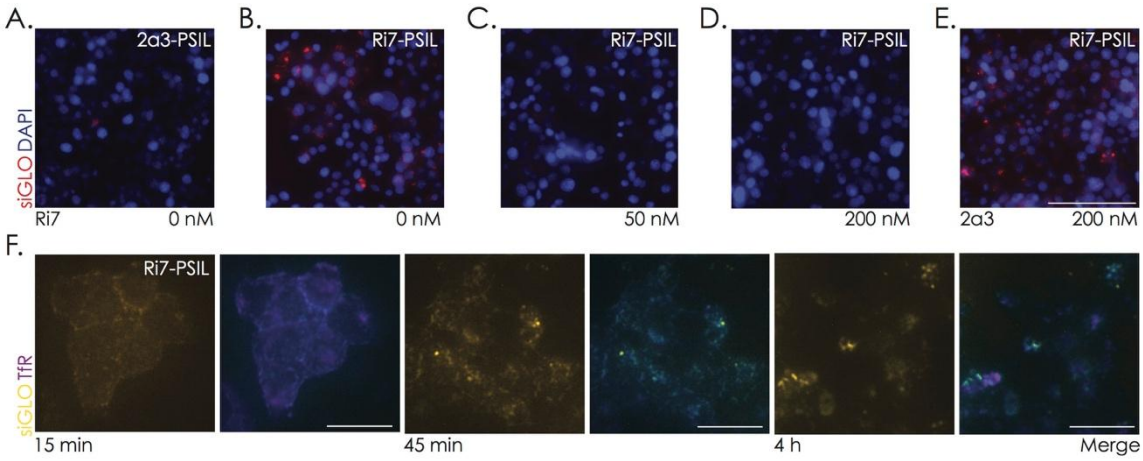

Figure 41: Ri7-PSILs uptake is mediated by TfR

N2A cells were seeded in 96-well plates containing DMEM and 10% FBS. Two day later, they were exposed to Ri7-PSILs or 2a3-PSILs containing siGLO Red (red) (500 nM). Representative micrographs following the incubation with **(A)** Control (2a3) PSIL, **(B)** Ri7-PSILs without or with a 30-min pretreatment with **(C)** 50 or **(D)** 200 nM of free Ri7 or **(E)** 200 nM of control IgG. Pretreatment with 50 or 200 nM of free Ri7 decreased the accumulation of siGLO following the incubation with TfR-targeted PICM, whereas a pretreatment with 200 nM of control IgG had no effect. **(F)** Representative images of the colocalization between Ri7-PSIL (yellow) and TfR (magenta) after 15, 45 min or 4 h of incubation with N2A cells demonstrating the involvement of TfR upon Ri7-PSIL internalization. Scale bars: A-E =100 µm, F = 25 µm.

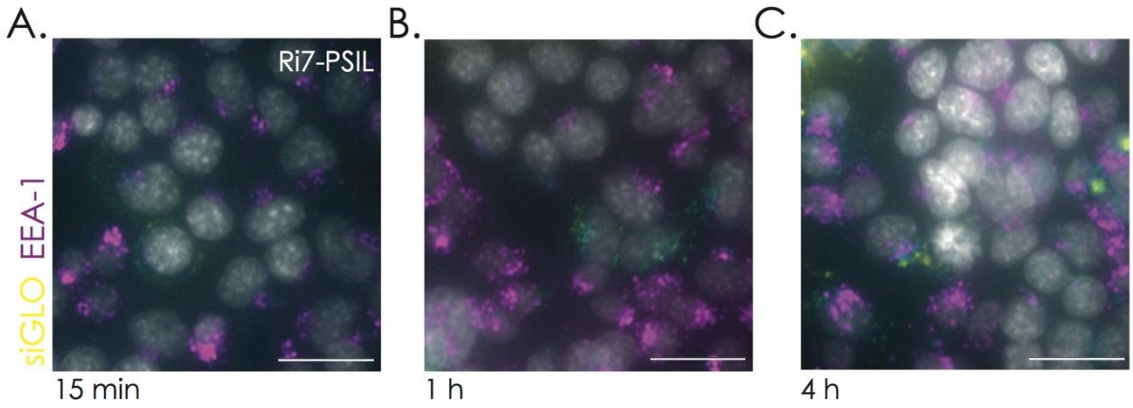

Figure 42: Ri7-PSIL enters endosomal pathway after internalization.

N2A cells were seeded in 96-well plates containing DMEM and 10% FBS. Cells were incubated with Ri7-PSILs containing siGLO (yellow) (500 nM) for **(A)** 15, **(B)** 45 min and **(C)** 4 h. Representative images of the colocalization between Ri7-PSILs (yellow) and endosomal marker EEA-1 (magenta) through time. Cell nuclei were counterstained with DAPI (white). Scale bar = 25 µm.

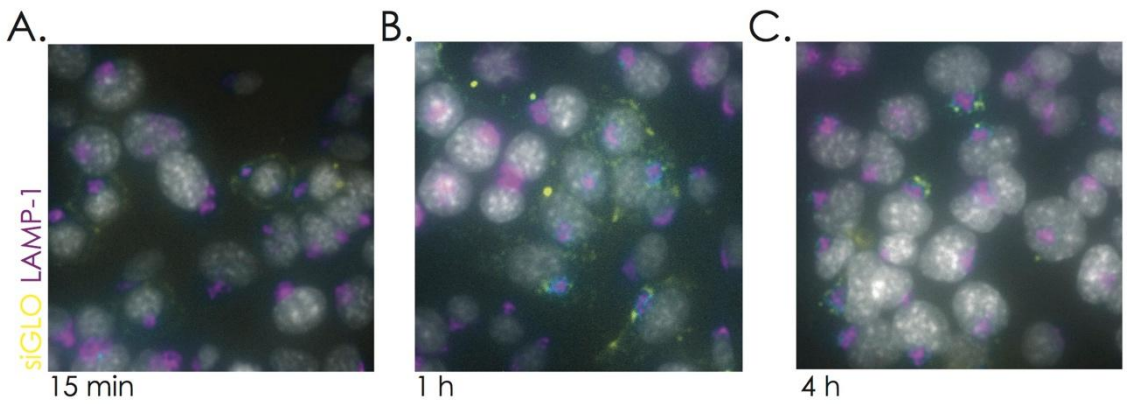

Figure 43: Ri7-PSIL are directed towards lysosomal compartments following endocytosis.
N2A cells were seeded in 96-well plates containing DMEM and 10% FBS. Cells were incubated with Ri7-PSILs containing siGLO (yellow) (500 nM) for **(A)** 15 min and **(B)** 4 h. Representative images of the colocalization between Ri7-PSILs (yellow) and lysosomal marker LAMP-1 (magenta) shows accumulation in the lysosome starting after 4 h of incubation on N2A cells suggesting potential lysosomal degradation. Cell nuclei were counterstained with DAPI (white). Scale bar = 25 µm.

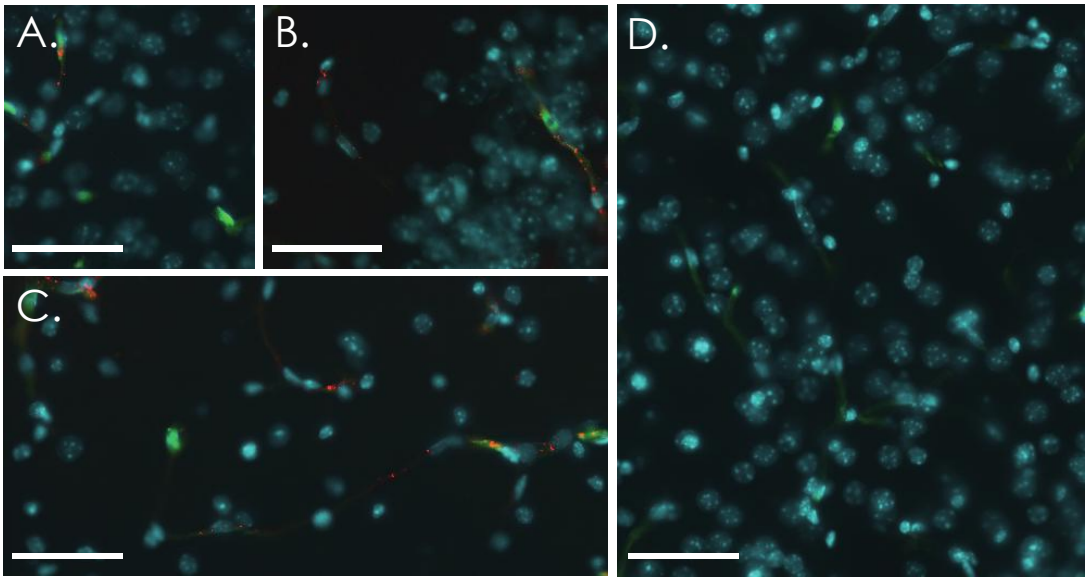

Figure 44: TfR-targeted PSILs deliver fluorescent DNA into BCECs of Tie2GFP mice

pDNA was labeled with AF546 and encapsulated into PSIL conjugated to the anti-TfR vector Ri7. Ri7-PSIL and 2a3-PSIL preparations were injected to Tie2GFP mice by intravenous injections. Animals were sacrifices 1 h later and processed for fluorescence microscopy. (A-C) Images showing the colocalisation of signal from AF546-pDNA (red) with GFP-expressing BCECs from Tie2GFP mice (green). Cell nuclei were marked with DAPI (cyan). (D) Absence of AF546 signal (red) following the administration of 2a3-PSIL in Tie2GFP mice. Scale bar = 50 µm

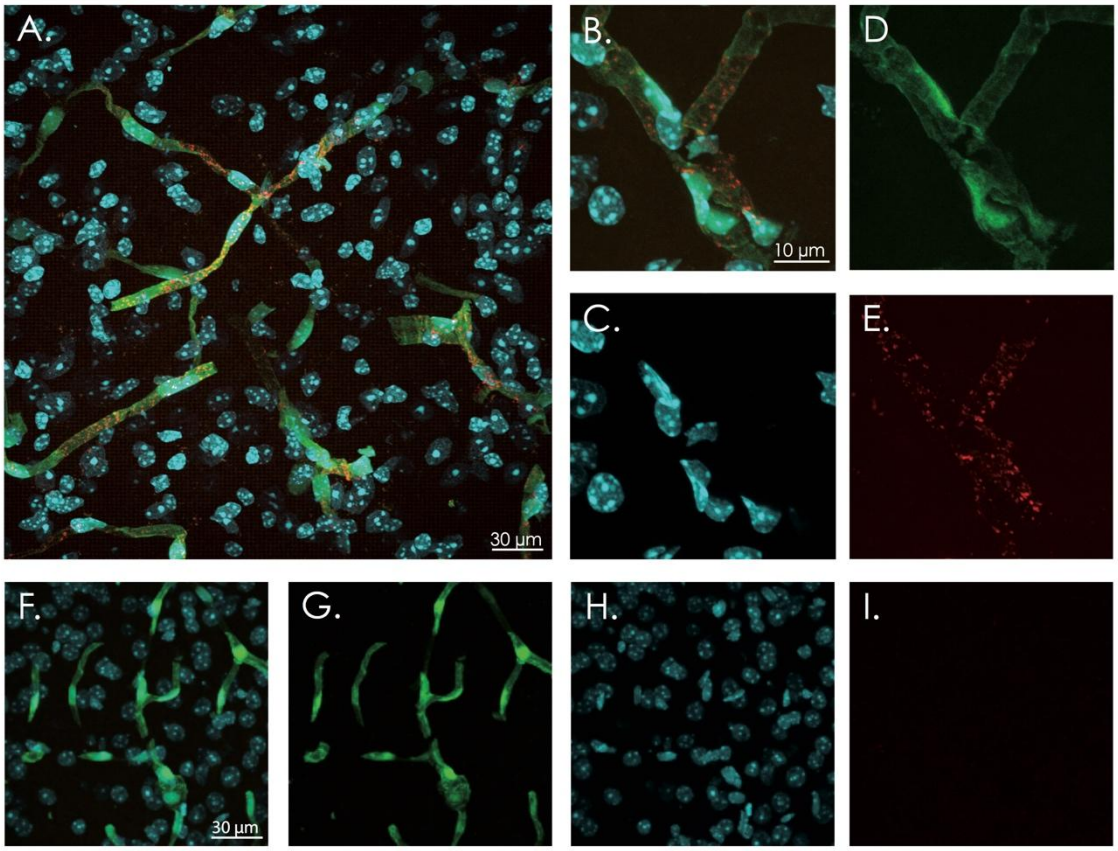

Figure 45: Delivery of fluorescent dsRNA analog to BCECs by PSILs.

Fluorescent dsRNA encapsulated into PSILs were administrated to Tie2GFP mice by intravenous injection. PSILs were conjugated to the anti-TfR Ri7 or to an isotypic vector 2a3. Animals were sacrificed 1 h following the injection and processed for fluorescent microscopy. **(A-E)** Confocal images showing the colocalization of signal from fluorescent dsRNA (red) with GFP-expressing BCECs from Tie2GFP mice (green). Cell nuclei were stained with DAPI (cyan). **(F-I)** Absence of signal from labeled siRNA (red) following the systemic administration of 2a3-PSIL in Tie2GFP mice. Images were taken with a confocal fluorescent microscope.

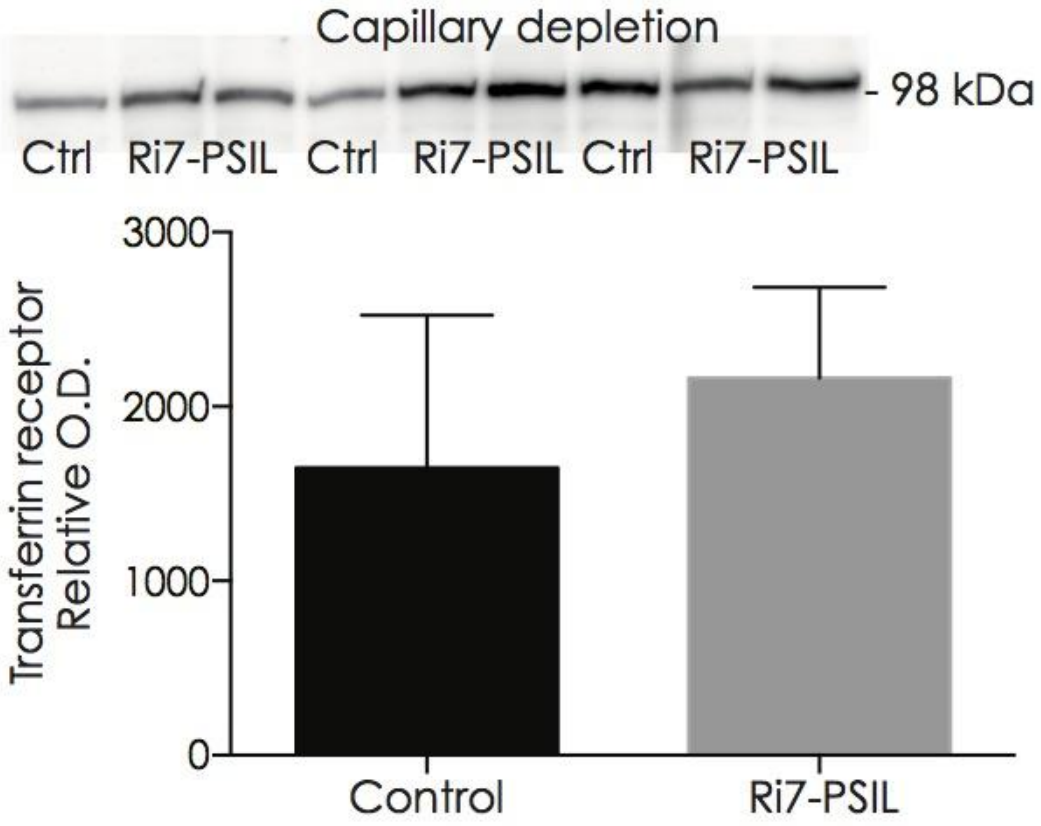

Figure 46: Administration of Ri7-PSIL does not affect the TfR levels *in vivo*.

Tie2GFP mice were injected twice a day for two days with Ri7-PSIL. Western blot quantification on detergent-soluble homogenates from concentrated capillary depletion fraction showed no significant reduction of TfR levels in mice injected repeatedly with Ri7-PSIL. Data represented are means ± SEM. Statistical comparison: Student t-test. n = 3 for control and 6 for Ri7-PSIL mice.