A standardised pre-clinical in-vitro blood-brain barrier mouse assay validates endocytosis dependent antibody transcytosis using transferrin receptor-mediated pathways

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Research Article

Keywords: In vitro BBB, Blood brain barrier, Transferrin receptor, TfR, Trancytosis, 3R

Posted Date: April 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1527031/v1

Abstract

The presence of the blood brain barrier (BBB) creates a nigh on impenetrable obstacle for large macromolecular therapeutics that need to be delivered to the brain milieu to treat neurological disorders. To overcome this, one of the strategies used is to bypass the barrier with what is referred to as a “Trojan Horse” strategy, where therapeutics are designed to use endogenous receptor-mediated pathways to piggyback their way through the BBB. Even though *in vivo* methodologies are commonly used to test the efficacy of BBB penetrating biologics, comparable *in vitro* BBB models are in high demand, as they benefit from being an isolated cellular system devoid of physiological factors that can on occasion mask the processes behind BBB transport via transcytosis. We have developed an *in vitro* BBB model (In-Cell BBB-Trans assay) that helps delineate the ability of modified large bivalent IgG antibodies, conjugated to the transferrin-receptor binder scFv8D3, to cross an endothelial monolayer grown on transwell inserts. Following the administrating of bivalent antibodies to the endothelial monolayer, a highly sensitive ELISA method is used to determine the concentration in the apical (blood) and basolateral (brain) chambers of the transwell system, allowing for the evaluation of apical recycling and basolateral transcytosis respectively. Our results show that antibodies conjugated to scFv8D3 transcytose at considerably higher levels compared to unconjugated antibodies in the In-Cell BBB-Trans assay. Interestingly, we are able to show that these results mimic *in vivo* brain uptake studies using identical antibodies. In addition, we are able to transversely section transwell cultured cells, allowing for the identification of biomarkers pertinent to transcytosis of the antibodies. Furthermore, studies using the In-Cell BBB-Trans assay revealed transcytosis of the transferrin-receptor targeting antibodies is dependent on endocytosis. In conclusion, we have designed a simple, reproducible In-Cell BBB-Trans assay that can be used to rapidly determine the BBB penetrating capabilities of transferrin-receptor targeting antibodies. We believe the In-Cell BBB-Trans assay can be used as a powerful, pre-clinical screening platform for therapeutic neurological pathologies.

Introduction

If Jesus was preaching to his disciples today, he might think about altering his ideas about who can be saved, by proclaiming, “A macromolecule has more chance of crossing the blood-brain barrier than for a rich man to enter the kingdom of God.” That is because blood-brain barrier (BBB) is one of the most tightly regulated physiological interfaces, regulated by physical, transport and metabolic barrier mechanisms to maintain the proper influx and efflux of metabolites to and from the brain (Abbott et al., 2010). The impermeable nature of the BBB importantly regulates the neuronal signalling microenvironment, but at the same time hinders the delivery of therapeutic agents. Aside from invasively delivering therapeutics to the neuronal microenvironment, current systemic delivery approaches typically rely on therapeutic interventions that can readily diffuse across the BBB and are no larger than 400 Da in size (Pardridge, 2012) or the blood-cerebrospinal fluid barrier (BCSFB), whose potential for drug delivery to the brain is currently the focus of multiple studies (Strazielle & Ghersi-Egea, 2016). This of course hampers and limits treatment strategies aimed at tackling neuronal disorders.

Many researchers worldwide are trying to find effective, safe therapeutic avenues to circumvent the molecular pathology evident in neurodegenerative diseases and other brain diseases. Protein based biological drugs are the fastest growing field in drug development, with a quarter of the newly approved drugs being proteins. Protein based biologics are uniquely adept in binding specifically to a disease target which enables them to treat diseases that small molecules cannot treat (Stanimirovic et al., 2018). The most recognized strategy to shuttle large biologics across the selectively permeable endothelial cell layer is to use the receptor-mediated endocytosis/transcytosis (RMT) pathways of the BBB (Jones & Shusta, 2007). The methodology relies on discovering receptors found on the apical side of the endothelial cell unit of the BBB that normally regulate the transport of essential nutrients and growth factors from the blood into the brain. Artificial, protein-based transporters that bind to these receptors can then be designed and these can in the ideal situation cross the endothelium via endocytosis/transcytosis into the extracellular environment of the brain. Since the endosome is large, one can recombinantly link therapeutic payloads to these transporters. We have successfully used such “Trojan horse” strategies to deliver intravenously injected antibodies against the pathological Ab protofibrils to the Alzheimer diseased brain in mice (Hultqvist et al., 2017). Our transporter bound the transferrin receptor that expressed promiscuously on the apical membrane of the endothelial cells, which is responsible for the transport of transferrin and iron to the brain parenchyma. The uptake compared to the antibody without the transporter was increased approximately 80 times. The same transporter has also been employed to similar effect to efficiently transport both antibody fragments and peptides into the brain (Fang et al., 2019; Rofo, Sandbaumhüter, et al., 2021; Rofo, Yilmaz, et al., 2021).

Alongside the advancement of novel methods to non-invasively deliver therapeutics to the brain, new pre-clinical *in vitro* analytical methodologies also need to be developed, to not only complement the necessary *in vivo* brain shuttling efficacy studies of the therapeutic in question, but also to abide by the directives of the EU concerning the reduction, replacement and refinement of animals used in research (European Parliament, 2010). The development of an *in vitro* cell culture model that mimics the *in vivo* blood brain barrier could be one such technique. It allows the user to pulse or load a therapeutic in the apical chamber of a cell-coated permeable support membrane (transwell), followed by the collection and analysis of media in the basolateral chamber at various time-points (referred to as the chase). In this set up, the apical chamber mimics the abluminal blood flow seen in the arterial and venous capillaries of the BBB, whereas the basolateral compartment resembles the luminal brain milieu. Ideally, if the therapeutic does have brain shuttling properties, its presence should be detectable within the basolateral chamber during the chase phase of the *in vitro* assay. There are several *in vitro* BBB model systems of varying complexity that are under development, which can be employed to mimic the *in vivo* BBB in certain physiological settings (Linville & Searson, 2021). However, the majority of the published *in vitro* BBB cell culture systems are focused on creating a model that closely resembles the *in vivo* BBB, rather than creating a cell culture system that can be used to test the brain shuttling efficacy of therapeutics. An *in vitro* model of the human BBB, based upon an adapted *in vitro* protocol developed using primary cultures of bovine brain microvessel endothelial cells (Raub & Newton, 1991), has been used to assess the role of pH on transcytosis rates of antibodies directed against potential brain shuttle receptors (Sade

et al., 2014). However, there is a lack of simple, descriptive mouse *in vitro* BBB systems that can be used pre-emptively to assess the efficacy of protein-based BBB penetrating therapeutics.

To this end we have developed the In-Cell BBB-Trans assay, a standardised mouse monolayer transwell culture system that can be used to assess the brain shuttling properties of antibodies conjugated to the transferrin receptor binder 8D3, which has been shown on numerous occasions to be an excellent BBB transporter in mice (Boado et al., 2009; Hultqvist et al., 2017; Zuchero et al., 2016). Utilising a “pulse-chase” strategy, the cell culture assay is streamlined so the entire assay can be completed within four-days, from initial plating of the cells to analysis. In addition, a highly sensitive enzyme-linked immunosorbent assay (ELISA) has been developed to work alongside the assay, detecting antibodies in cell medium down to a concentration as low as 0.5 pM. Furthermore, using transverse cryosections of the transwell membranes and subsequent immunohistochemical staining techniques, affords the user the ability to detect the molecular orchestrators of antibody transcytosis in an apical/basolateral orientation. Initial findings obtained using the In-Cell BBB-Trans assay shows a significant increase in transcytosis of scFv8D3 conjugated antibodies compared to unconjugated antibodies, as well as verifying the requirement of endocytosis pathways in transferrin receptor mediated transcytosis of scFv8D3 conjugated antibodies (Fig. 1). Together, the described In-Cell BBB-Trans assay provides a robust methodology for quickly and efficiently validating possible transferrin receptor related brain shuttling antibodies, as well as providing a platform for delineating the molecular mechanisms behind these processes.

Materials And Methods

Design, expression and purification of monoclonal bivalent antibodies and proteins

The four bivalent monoclonal IgG antibodies and proteins used in the experiments were designed, expressed and purified according to earlier published work (Fang et al., 2017; Hultqvist et al., 2017). The RmAb158 monoclonal antibodies selectively bind to Ab protofibrils (Englund et al., 2007), whereas the RmAb2G7 monoclonal antibodies selectively binds to High mobility group box 1 proteins (HGMB1) (Lundbäck et al., 2016). In short, the heavy and light chain scFv8D3 transferrin receptor transporter variable region sequence (Boado et al., 2009) was connected to the C-terminus of the RmAb2G7 or RmAb158 light chain with in-house designed linkers (APGSYTGSAPG and APGSGTGSAPG respectively). Figure 2 shows cartoon representations of the antibody design, showing the location of the conjugated scFv8D3 in the modified antibodies. The four recombinant antibodies were expressed using Expi293 cells (Thermofisher) transiently transfected with pcDNA3.4 vectors using polyethylenimine (PEI) as the transfection reagent. All antibodies were purified on a protein G column (Cytiva) and eluted with an increasing gradient of 0.7% Acetic Acid. The buffer was exchanged to PBS (Gibco) immediately after elution and the protein concentration was determined at A280.

In-Cell BBB-Trans assay

A murine cerebral endothelial cell line (cEND) obtained from Applied Biological Materials

(Passages 15–48) was grown on rat tail Collagen Type I (Sigma – 50 µg/ml) coated 75cm^2 culture flasks (Sarstedt) in complete cEND medium (DMEM supplemented with 10% FBS, 1X non-essential amino acids, 1X Glutamax, 1 mM sodium pyruvate and 10 U/ml Penicillin/Streptomycin – all media and supplements were purchased from Gibco) at 37^{o}C and 5% CO_2. Optimal media volumes were calculated to be 125 µl and 800 µl for apical and basolateral chambers respectively. For all transcytosis assays, Bio-One Thincert™ transparent (2 x10^6 pores/cm^2) and translucent (1 x10^8 pores/cm^2) PET membranes with high density 0.4 µm pores were used in 24-well cell culture plates (Greiner). Apical chambers of the Greiner hanging inserts were coated with Collagen type IV (Fisher scientific – 20 µg/cm^2) followed by Fibronectin (Sigma – 20 µg/cm^2), each incubation lasting for one hour at 37^{o}C and 5% CO_2. The cEND cells were plated at a density of 9 x 10^4 cells in the apical chamber on day one and were re-fed no earlier than 4-hours later with cEND differentiation medium (2% FBS, 1X non-essential amino acids, 1X Glutamax, 1 mM sodium pyruvate and 10 U/ml Penicillin/Streptomycin). On day 4, the Greiner membranes were pulse-incubated apically with 13.3, 133.3 or 266.6 nM of monoclonal bivalent antibodies in serum-free conditions (no FBS) at 37°C and 5% CO2 for 15-minutes or one-hour. Volumes used for the pulse apical and basolateral chambers, 75 ml and 400 ml respectively, were collected to corroborate the starting concentration of the antibodies used and determine the barrier properties of the cEND cells (Pulse samples). The monolayers were washed at room temperature in serum-free medium apically (400 ml) and basolaterally (800 ml) three times, with the final wash collected to monitor efficiency of removal of the unbound antibodies (Wash samples). Serum-free medium was added to the apical (75 ml) and basolateral (400 ml) chambers. The cultures were incubated at 37°C and 5% CO_2 for four or six hours, upon which time entire apical and basolateral volumes were collected to assess the recycling and transcytosis of the antibodies into the apical and basolateral chambers respectively (Chase samples). A representation of the In-Cell BBB-Trans assay can be seen in Fig. 3. Light cell microscopy images of cEND cells plated on transparent transwells were taken using a Visiscope® inverted light microscope (VWR) mounted with a Moticam 1080 BMH digital camera (VWR). Image processing and scale bars were generated using ImageJ software (Schneider et al., 2012).

Sandwich ELISA analysis of media samples from the transcytosis assay

A 96-well ELISA plate was coated with 1/5000 Goat-anti Mouse IgG, F(ab')$_2$ fragment specific antibody (JacksonImmunoResearch) diluted in PBS and incubated at 4°C overnight. The wells were blocked with 1% BSA/PBS for one-hour at room temperature on a 500-rpm shaking platform, followed by washing five-times with 0.05% Tween 20/PBS using a Tecan Hydroflex microplate washer. Diluted and undiluted apical and basolateral samples from the transcytosis assay, along with known standard concentrations of monoclonal bivalent antibodies, were added to the wells and incubated for two-hours at room

temperature on a 500-rpm shaking platform. The wells were washed as previously described and subsequently incubated with 1/5000 Goat anti-Mouse HRP (Sigma) diluted in 0.1% BSA/0.05% Tween 20/PBS for one-hour at room temperature on a 500-rpm shaking platform. Following a final wash cycle, the wells were developed with K-Blue®TMB Aqueous substrate (Neogen) at room temperature according to the manufacturer's recommendations, using 1 M H_2SO_4 to stop the reaction (approximately 5–8 minutes following the addition of TMB). Absorbance readings at 450 nm were measured immediately using a FLUOstar® Omega ELISA plate reader (BMG Labtech) and the data was analysed using Omega Control (BMG Labtech) and Prism 9 for macOS. Statistical analysis between indicated populations was performed using an unpaired non-parametric Mann-Whitney test and the minimal accepted significance level was $P \leq 0.05$.

Immunohistochemistry of cEND cell plated translucent transwells

Following the conclusion of a pulse-chase experiment, cEND coated translucent transwells were rinsed two times with PBS and then fixed for 10 minutes in 4% paraformaldehyde (VWR). Following a further three washes with PBS, the transwell membranes were carefully removed using a scalpel blade and mounted vertically in 6% gum tragancanth (Sigma) on a 20 mm cork disc (Thermo Fisher Scientific). The mounted transwell was snap-frozen in dry-ice cooled isopetane and immediately stored at -80°C. Using a CryoStar NX70 (ThermoScientific) cryostat set to -20°C, eight-micron cryosections were cut and mounted on Thermofrost® Plus (Thermo Fisher Scientific) glass cover slides. Individual sections were isolated with a wax pen, rinsed three times with Tris-Buffered Solution (TBS) and permeabilised for ten minutes at room temperature with 0.1% Triton X-100/TBS. Sections were rinsed in 0.05% Tween 20 (wash buffer - diluted in TBS) three times, followed by a thirty-minute block incubation at room temperature in 10% Bovine Serum Albumin (BSA - diluted in TBS). Sections were rinsed in wash buffer three times, followed by an overnight incubation at 4°C with primary antibodies diluted in 10% BSA/TBS (1/100 Rat anti-Transferrin receptor (NovusBio), 1/50 Goat anti-CD31 (R&D Systems) or 1/100 Rabbit anti-Rab5 (Abcam)). Sections were rinsed in wash buffer three times, followed by a one-hour room temperature incubation with 1/500 host-targeted fluorescently labelled antibodies (Goat anti-Rat Alexa 488, Donkey anti-Goat Alexa 488, Goat anti-Rabbit Alexa 555 or Donkey anti-Mouse 555 (Thermo Fisher Scientific)). Sections were rinsed in wash buffer three times and mounted with a glass coverslip in Fluoromount-G™ medium (Thermo Fisher Scientific) supplemented with 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI).

Epi-fluorescent images were taken using an Olympus DP73 fluorescent microscope with cellSens Dimension software and processed for publication using ImageJ software. Confocal images were taken using a LSM700 microscope with Zeiss Zen software and further analysed using deconvolution software Huygens (Scientific Volume Imaging). ImageJ software was used to create an audio video interleave (AVI) movie from individual deconvoluted z-stack confocal images and perform further image processing for publication.

Labelling of antibodies with 125-idoine

Labelling of the antibodies with 125-iodine was performed as previously described (Rofo, Yilmaz, et al., 2021). Equimolar amounts of RmAb2G7 and RmAb2G7-scFv8D3 were each labelled with 8 MBq of 125-iodine (Perkin Elmer Inc., Waltham, MA), resulting in a labelling yield of 70%.

Brain uptake studies in wild-type mice

C57Bl/6 mice (3 months of age) were used in this study. The mice were housed in an animal facility at Uppsala University, with free access to water and food and under controlled temperature and humidity. Experimental procedures were approved by the Uppsala County Animal Ethics Board (#5.8.18–13350/17). Mice were intravenously injected via tail vein with a tracer dose of 0.05 mg/kg of I^{125}-RmAb2G7 (n = 6) or I^{125}-RmAb2G7-scFv8D3 (n = 6). At two-hours and twenty-four-hours post injection, three mice were euthanized by transcardial perfusion with 0.9% NaCl. Brains were dissected and radioactivity was measured using Wizard 2470 gamma counter (Perkin Elmer Inc., Waltham, MA) as described previously (Rofo, Yilmaz, et al., 2021). Statistical analysis between indicated populations was performed using an unpaired parametric Welch t-test and the minimal accepted significance level was $P \leq 0.05$.

Preparation and use of Dyngo4a™ in the pulse-chase assay

Dyngo-4a™ (Abcam) was resuspended in sterile-filtered DMSO to yield a 1000X stock concentration of 30 mM. Thirty minutes prior to pulsing the monoclonal bivalent IgG antibodies on the cEND cell coated Bio-One Thincert™ translucent transwells, a volume of 30 mM Dyngo-4a™ was added to the apical and basolateral compartments of the cell culture to provide a working concentration of 30 mM. Following the pre-incubation with Dyngo-4a™, the apical and basolateral compartments in the pulse and chase phases of the assay (previously described in the Materials and Methods) were supplemented with 30 mM Dyngo-4a™. Control cultures were performed at the same time, with identical passages of cEND cells, replacing Dyngo-4a™ with sterile-filtered DMSO (Sigma) at comparable time-points.

Results

Translucent transwell matrices are optimal for transcytosis studies

The development of the mouse In-Cell BBB-Trans assay, capable of monitoring transferrin receptor mediated transcytosis, required a robust, standardised methodology that allowed for a stringent quality control and washing protocol, so recycling and transcytosis could be quantitatively measured. A Greiner Bio-One® transwell-based culture system was chosen as the optimal growth matrix, based upon a previous study that showed immortalised endothelial cells optimally grew on this type of matrix compared to other named brands (Eigenmann et al., 2013). A In-Cell BBB-Trans assay was employed to load a monolayer of mouse cEND cells (Fig. 3), with monoclonal bivalent antibodies conjugated with a transferrin receptor binder (8D3) that has previously been shown to cross the mouse BBB *in vivo* (Boado et al., 2009; Hultqvist et al., 2017; Zuchero et al., 2016) (Fig. 2). Unlike other types of *in vitro* BBB models,

the development of a system representing a tight physiologically comparative barrier as seen *in vivo* was not the priority. Instead, the In-Cell BBB-Trans assay relies heavily on quantitively assessing apical recycling or basolateral transport of the loaded antibodies. In order for the In-Cell BBB-Trans assay to function, a rigourous, quantitative quality control was employed to accurately assess the concentration of the antibody pulsed, as well as the quantity of the antibody remaining in the apical and basolateral compartments following the one-hour pulse and washing protocol. To this end, a highly sensitive ELISA protocol was developed that was able to detect mouse IgG antibodies down to 0.5 pM, allowing for a sensitive quantitative analysis to be performed (Fig. 4A).

Initial studies were carried out on 24-well 0.4 µM transparent pore transwells (2 x 10^6 pores/cm^2), which allowed for visualisation of the cEND cell monolayer during the preparatory differentiation phase (Fig. 4B) and following the chase phase (Fig. 4C) of the experiment. Even though the cells looked healthy growing on the transwell matrix following the chase phase of the experiment, the bivalent antibodies conjugated with and without the 8D3 transporter were undetectable in the basolateral compartment (Supplementary Fig. 1A). The apical recycling was unaffected however, with a significant increase in the concentrations of RmAb2G7-scFv8D3 in the apical compartments compared to RmAb2G7, whether a 15-minute or 1-hour pulse was used. In addition, the concentration of antibody in the apical and basolateral pulse and wash samples indicated that the correct concentration of antibody was pulsed, the cells were performing an adequate barrier function and the wash phase was removing unbound/non-loaded antibody from the cell culture system prior to the chase phase. To test whether the lack of basolateral transport was due to steric hinderance brought about by too few pores present in the transparent transwell membrane, a structurally identical 0.4 µM translucent pore membrane (1 x 10^8 pores/cm^{-2}), containing 50-times more pores, was used in the In-Cell BBB-Trans assay. Using the translucent transwells definitely improved the basolateral transport of the antibodies (Supplementary Fig. 1A), showing an increased, but not a significant increase in concentration of RmAb2G7-scFv8D3 in the basolateral chase compartments compared to RmAb2G7. Based on these results, it was decided that the translucent transwell membranes would be used for further characterization studies in the In-Cell BBB-Trans assay.

A monolayer of cEND provides a strong barrier against antibodies

As mentioned earlier, the main priority of the In-Cell BBB-Trans assay was to create a standardised model of apical recycling and basolateral transcytosis, rather than creating an impermeable, physiologically comparable barrier. However, it was essential that the *in vitro* model does not allow everything to escape through the monolayer of cEND cells during the pulse phase of the assay. Multiple repetitions of the In-Cell BBB-Trans assay revealed that a large proportion of the pulsed antibody concentration remained within the apical compartment of the transwell, whereas around 1–5% of the original antibody concentration travels through to the basolateral compartment (Fig. 5A). To assess the barrier capabilities of the cEND monolayer, the same experiment was repeated, without the addition of the cEND cells to the transwell. In the space of the one-hour pulse, approximately 30–33% of the original antibody

concentration diffused to the basolateral compartment, representing an approximate ten- to twenty-fold increase in transport across the transwell membrane compared to when there is a plated monolayer of cEND cells present (Fig. 5B). This result provides evidence that the cEND cells provide a strong barrier against large macromolecules.

Transverse, high-resolution images through the cEND monolayer provide greater analytical capabilities

One of the drawbacks of using translucent transwell membranes is the lack of optical transparency, making microscopic viewing of the cells impossible. However, it is possible to mount the cEND coated transwells in such a fashion that ultrathin transverse cryosections can be made through the cells, removing the transwell optical barrier and providing cross-sectional cellular images in an apical to basolateral orientation. One can immunofluorescently label the cell sections, highlighting pertinent biomarkers involved with the processes of transferrin receptor mediated transcytosis. Figure 6A shows a basic epifluorescent image of a cEND monolayer section, co-labelled with transferrin receptor (green) and DAPI (blue) to visualise the nuclei, clearly showing the cEND cells assembled on top of the transwell membrane in a monoloayer. Higher resolution deconvoluted confocal images can further delineate biomarkers involved with transcytosis, as can be seen in Fig. 6B, where a polarised staining of transferrin receptor (green) can be seen predominantly localised to the apical membrane of the cell, whereas the early endosome marker Rab5 (red) can be localised in the vicinity of transferrin receptors and more promiscuously distributed throughout the cell cytoplasm. Supplementary Movie 1 shows a z-stack compilation through cross-sections of cEND cells labelled with the endothelial cell marker CD31 (green), where one can visualise the pulsed RmAb2G7-scFv8D3 antibodies (labelled in red) dispersed throughout the cytoplasm and adjacent to the membrane of the cEND cells. In summary, the described immunohistochemistry technique can be adapted and applied to the In-Cell BBB-Trans assay setup, allowing for a greater in-depth molecular delineation of the transcytosis associated pathways.

The In-Cell BBB-Trans assay can be used to identify transferrin receptor mediated transcytosis

Pulsing 133.3 nM bivalent antibodies to cEND cells grown on translucent transwells provided strong evidence that transcytosis could be quantified in the In-Cell BBB-Trans assay. To further improve the experimental setup, a lower concentration of antibody was pulsed to reduce the possible cross-linking and endosomal retention of the transferrin receptor (Marsh et al., 1995). We firstly tested monoclonal antibodies that selectively target Ab protofibrils (RmAb158 and RmAb158-scFv8D3). Figure 7A comprehensively shows a significant increase in apical recycling and basolateral transcytosis in the RmAb158-scFv8D3 pulsed cultures compared to RmAb158. These results closely mimic previously published *in vivo* data by our group (Hultqvist et al., 2017), which showed an 80-fold increased brain uptake following intravenous administration of RmAb158-scFv8D3 in C57Bl/6 mice compared to RmAb158. To show that the process we were observing in our *in vitro* model system was down to the

action of 8D3 alone and not some non-specific pathway based on the antibodies selective binding capabilities, we next tested RmAb2G7, an antibody designed to targets HGMB1. Once again, Fig. 7B shows a very similar outcome when comparing apical recycling and basolateral transcytosis in RmAb2G7-scFv8D3 pulsed cells compared to RmAb2G7. Interestingly, when we performed an *in vivo* brain uptake study with these antibodies in C57Bl/6 mice, we showed comparable results to our *in vitro* data, with a significant increase in brain uptake with RmAb2G7-scFv8D3 compared to RmAb2G7 (Fig. 7C). These results show that the In-Cell BBB-Trans assay mimics brain uptake of transferrin receptor targeted bivalent antibodies *in vivo.* Further studies using elevated concentrations of pulsed bivalent monoclonal antibodies, and an increased chase phase (six-hours instead of four), also showed clear transport disparities between antibodies conjugated with and without the scFv8D3 transporter. Figure 7D shows the apical recycling and basolateral transcytosis is significantly higher in RmAb158-scFv8D3 compared to RmAb158, at pulsed concentrations of 133.3 and 266.6 nM. In addition, note the elevation of the RmAb158 levels in both the 133.3 and 266.6 nM pulsed cultures (Fig. 7D) compared to 13.3 nM (Fig. 7A), which could indicate that the antibody is migrating across the cEND monolayer via alternate non-transferrin receptor mediated transport pathways at higher concentrations. Based on these findings, in order to ascertain the antibodies' ability to undergo specific transferrin receptor mediated transcytosis, it would be ideal to use the lower pulse concentration of 13.3 nM.

Transferrin receptor mediated transcytosis requires endocytosis

Transferrin is an iron-binding protein, which binds to the transferrin receptor and enters the cell through endocytosis mediated processes (Mayle et al., 2012). The precise mechanistic details on the role of endocytosis in transferrin receptor-mediated transcytosis of administered antibodies remains to be elucidated and is still up for debate. To gain insight into whether endocytosis is necessary for transcytosis of pulsed 8D3-conjugated antibodies, an endocytosis inhibitor Dyngo4a™ (30 mM) was added to the cEND monolayer 30 minutes prior to, and during, the pulse phase, as well as during the duration of the chase phase of the In-Cell BBB-Trans assay. As can be seen in Figs. 8A and B, Dyngo4a™ works by inhibiting dynamin, a GTPase essential for completing endocytosis in eukaryotic cells (Mccluskey et al., 2013). Interestingly, as shown in Fig. 8C, when Dyngo4a™ was added to the culture, the apical recycling of RmAb2G7-scFv8D3 was unaffected, whereas the level of basolateral transcytosis was significantly reduced compared to cells treated with the DMSO carrier. These results primarily indicate that when endocytosis is inhibited, the antibody bound to the transferrin receptor cannot be internalised, resulting in reduced transcytosis. Being that the antibody bound transferrin receptor cannot be internalised, the results also indicate that apical recycling we are observing in our In-Cell BBB-Trans assay is more likely due to antibodies being released from the transferrin receptor at the surface of the cell during the chase phase of the assay, rather than transferrin receptor bound antibodies entering the cells and being recycled to the apical compartment using the canonical transferrin receptor recycling pathways (Mayle et al., 2012).

Higher cEND passages reduce the sensitivity, but not the specificity of the in vitro assay

To ensure the In-Cell BBB-Trans assay is sustainable and can be used after multiple passages of cEND cells, the In-Cell BBB-Trans assay was performed using cEND cells at lower and higher passages to compare the apical recycling and basolateral transcytosis of an 8D3 conjugated bivalent monoclonal antibody. Figure 9 shows that both apical recycling and basolateral transcytosis is significantly reduced in passage 48 cEND cells compared to passage 11. These results go some way to explaining the discrepancy observed between the absolute concentration of apical recycling and basolateral transcytosis levels between the In-Cell BBB-Trans assay results shown in Figs. 7 and 8, as different cEND passages were used to run each experimental set. This is clearly exemplified when comparing the reduced apical recycling and basolateral transcytosis levels of 13.3 nM RmAb2G7-scFv8D3 pulsed passage 29 cEND cells in Fig. 8 compared to 13.3 nM RmAb2G7-scFv8D3 pulsed passage 15 cEND cells in Fig. 7B. In summary, even though the level of apical recycling and basolateral transcytosis sensitivity is reduced as the passage number increases, it is still possible to run comparative transferrin receptor mediated transport analysis to ascertain transcytosis efficacy, as long as the assay is completed using the same passage of cells.

Discussion

The need for designing therapeutic strategies that can safely cross the BBB to treat different neurological maladies is of the utmost importance, and coinciding with the development of these strategies, robust methodologies must be in place in order to pre-clinically test any promising candidates. The requirement for pre-clinical testing of any biologic therapeutic cannot forgo *in vivo* systems when evaluating safety and efficacy. However, aside from the cost and inherent difficulties of planning and carrying out animal experimentation to test biopharmaceutical treatment strategies, it is our duty to seriously consider the reduce, replacement and refinement protocols for all aspects of pre-clinical *in vivo* testing (Kirschner, 2021). Furthermore, *in vitro*-based BBB models are not hampered by the behavioural or systemic effects that drive BBB disruption in *in vivo* models, making it easier to define and identify key cellular/molecular players, targets and regulators of transport across the BBB (Williams-Medina et al., 2021). With this said, the implementation of simple *in vitro* systems that can test multiple biologics and physiological-like conditions would definitely provide a good foundation to start with when assessing the molecular efficacy of BBB penetration.

We have developed such an In-Cell BBB-Trans assay that can effectively assess the BBB penetrance ability of large IgG antibodies using transferrin-receptor mediated transcytosis pathways. The methodology of using transwell systems to monitor BBB transport is not a novel concept. However, as amazing as they are, a large proportion of these *in vitro* systems rely on creating a physiological like-for-like model, focussing heavily on creating a cellular barrier that does not allow even the smallest of molecules to penetrate it (Helms et al., 2015). The main ideology of the In-Cell BBB-Trans assay differs

somewhat, by focussing more on the removal of unbound or background pulsed antibodies, while maintaining the cells in a minimally disturbed and healthy state, which is conducive for their ability to perform physiological tasks such as transcytosis of bound antibodies. With that said, the cell's ability to act as a formidable barrier is not forsaken and we have shown that a monolayer of cultured cEND cells massively reduces the transport of the antibody from the upper apical chambers of the transwell system into the lower basolateral chambers (Figs. 5A and B). Using this simple 24-well transwell system, which lends itself to testing multiple targets and repetitions at any one time, we can definitively show the specific transport of modified bivalent antibodies conjugated to the transferrin receptor binder scFv8D3 when compared to unmodified antibodies (Figs. 7A, C and D). In addition, we also show identical outcomes to the In-Cell BBB-Trans assay, when testing these antibodies using *in vivo* brain uptake studies (Fig. 7C and (Hultqvist et al., 2017)). These results indicate that the In-Cell BBB-Trans assay closely mimics the brain uptake of bivalent antibodies *in vivo* and can be employed as a more translatable model system in terms of drug development and preclinical settings, thereby reducing and refining the *in vivo* burden of testing BBB penetrating therapeutic targets.

Even though it is known that canonical transferrin transport into cells occurs via clathrin-mediated endocytosis pathways via the transferrin-receptor (Mayle et al., 2012), little is known as to whether antibodies that target the transferrin-receptor enter in a similar fashion. A previous study, using an elegant *in vitro* BBB organoid array system, showed that transcytosis of a monovalent antibody targeting the human transferrin receptor is dependent upon clathrin-mediated endocytosis (Simonneau et al., 2021). We can quantitatively confirm this finding, as the level of transcytosis of RmAb2G7-scFv8D3 is significantly reduced when endocytosis is inhibited in our In-Cell BBB-Trans assay using the dynamin inhibitor Dyngo 4a™ (Fig. 8). Even though the translucent transwells used in our In-Cell BBB-Trans assay do not lend themselves easily to microscopic studies, we have developed a simple methodology for mounting and sectioning cEND cells grown on transwell membranes, allowing immunohistochemical analysis of biomarkers pertinent to transcytosis pathways. Along with the In-Cell BBB-Trans assay, this technique provides a transverse view of the cell monolayer making the identification of molecular markers important for transcytosis easier to identify in relation to the apical and basolateral orientation of the cell (Fig. 6 and Supplementary Movie 1).

The caveat of using *in vitro* culture systems is the progressive changes that can occur at a cellular level as the passage number increases. A way around this problem is to use embryonic stem cells (ES) and induced pluripotent stem cells (iPS) to produce *in vitro* BBB models, as they can be expanded indefinitely and they are capable of differentiating into all the derivatives of the three germ layers, thus removing the possible down-regulation of physiological cellular functions with time (Romito & Cobellis, 2016; Vazin & Freed, 2010). However, the elevated ethical discussions of using such cell systems, along with the difficulty in generating homogenous populations of cells, provides a hinderance to using such cell systems to produce large-scale *in vitro* BBB models. Our described In-Cell BBB-Trans assay relies on cells that can be easily sourced, cultured without the need for specialist media and can be used at a range of passages to determine transferrin-receptor mediated transcytosis. In addition, the entire In-Cell BBB-Trans assay is expedient, taking a maximum of four to five days to go from plating the cells on the transwell

membrane to obtaining quantitative data ready for analysis. We show that the cEND cells used in our described In-Cell BBB-Trans assay show apical recycling and basolateral transcytsosis at elevated passages, albeit at reduced levels compared to lower passages (Fig. 9). However, as long as the pertinent positive and negative controls are added to performed studies, elevated passages do not hinder obtaining comparative quantitative results that would help delineate the efficacy of transcytosis through the cEND monolayer.

Conclusion

We have developed a rapid, standardised and reproducible In-Cell BBB-Trans assay that is capable of discerning the transcytosis capabilities of bivalent antibodies using transferrin-receptor mediated transcytosis pathways, uncannily mimicking the findings of brain uptake studies performed in wild-type mice. The cell culture setup can be manipulated to investigate different physiological settings and can possibly be used to test alternate BBB transporter pathways. In short, the In-Cell BBB-Trans assay provides a platform for pre-clinical screening for therapeutic intervention strategies towards neurodegenerative and neurological pathologies.

Declarations

Author contributions

JIM, HL and GH designed the project. JIM and SS have been responsible for developing the In-Cell BBB trans assay. JIM and AP performed the In-Cell BBB trans assays. JIM performed the cEND immunohistrochemistry experiments. NM, FR and CUY produced, analyzed and radio-labelled antibodies, along with conducting the *in vivo* experiment. JIM analyzed the results together with HL and GH. JIM and GH wrote the manuscript with valuable inputs from all the co-authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures described in this paper were approved by the Uppsala County Animal Ethical Committee following the ethical guidelines and having the ethical permission numbers: 5.8.18-13350/17 and 5.8.18-20401/20.

Competing interests

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding

This work was supported by grants from Swedish Research Council, Hedlunds stiftelse, Åke Wibergs stiftelse, Åhlén-stiftelsen, Jeanssons stiftelser, Magnus Bergvallsstiftelse, Vinnova, Alzheimerfonden, Torsten Söderbergs stiftelse and Byggmästare Olle Engqvist stiftelse.

Acknowledgements

Biorender.com was used to create illustrations. Radiochemistry and animal work in this study was performed at the SciLifeLab Pilot Facility for Preclinical PET-MRI, a Swedish nationally available imaging platform at Uppsala University, Sweden, financed by the Knut and Alice Wallenberg Foundation.

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Figures

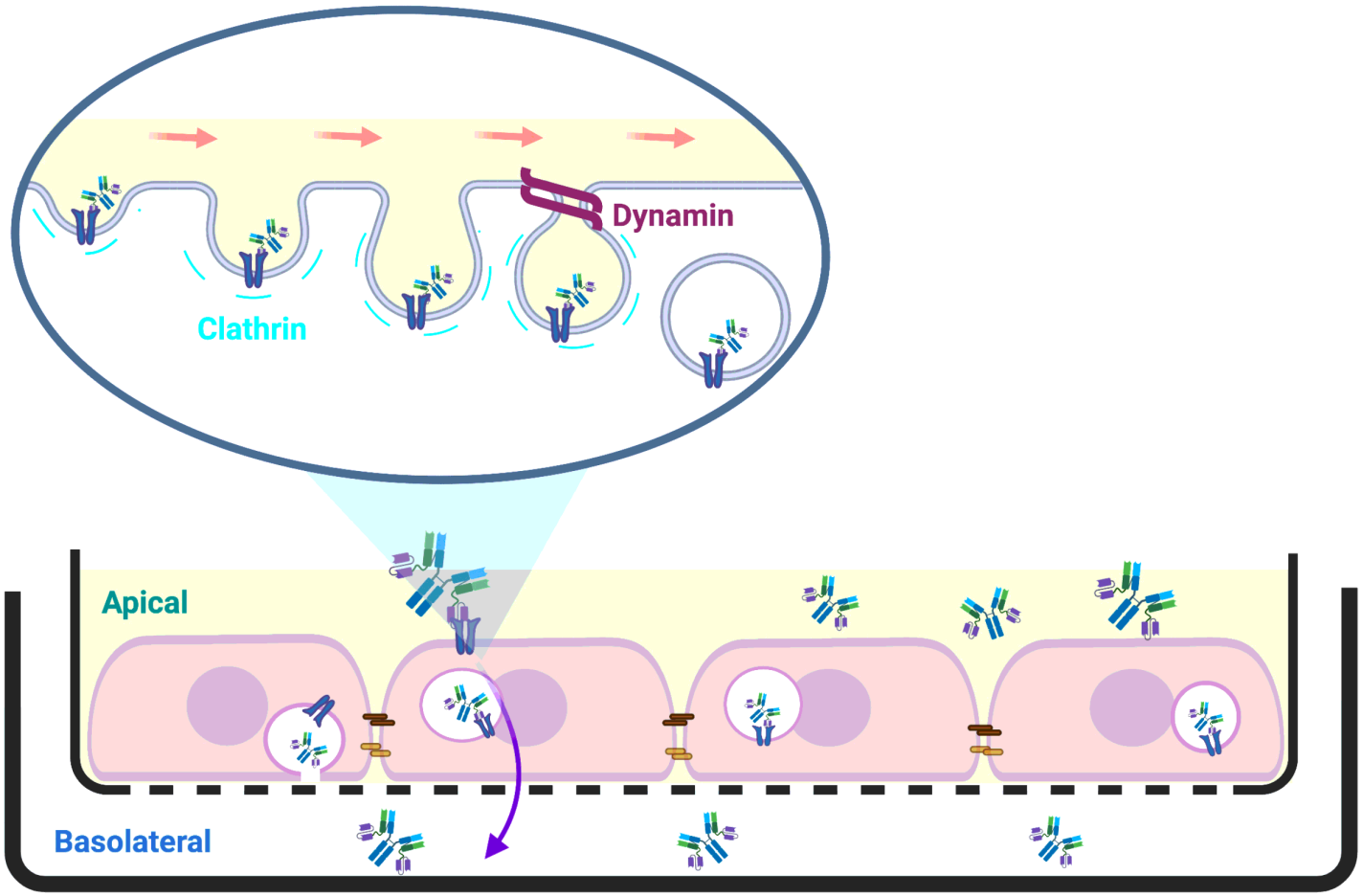

Figure 1

A cartoon summary depicting a single transwell setup of the In-Cell BBB-Trans assay. The cartoon shows a monolayer of mouse endothelial cells being pulsed with transferrin receptor binding 8D3 conjugated bivalent antibodies. The antibodies can be seen binding to the transferrin receptor, undergoing endocytosis into the cell (the process of which is visualised in the magnified image insert) and travelling from the apical (blood mimicking) compartment through to the basolateral (brain milieu mimicking) compartment via transcytosis pathways. Illustration created with Biorender.com.

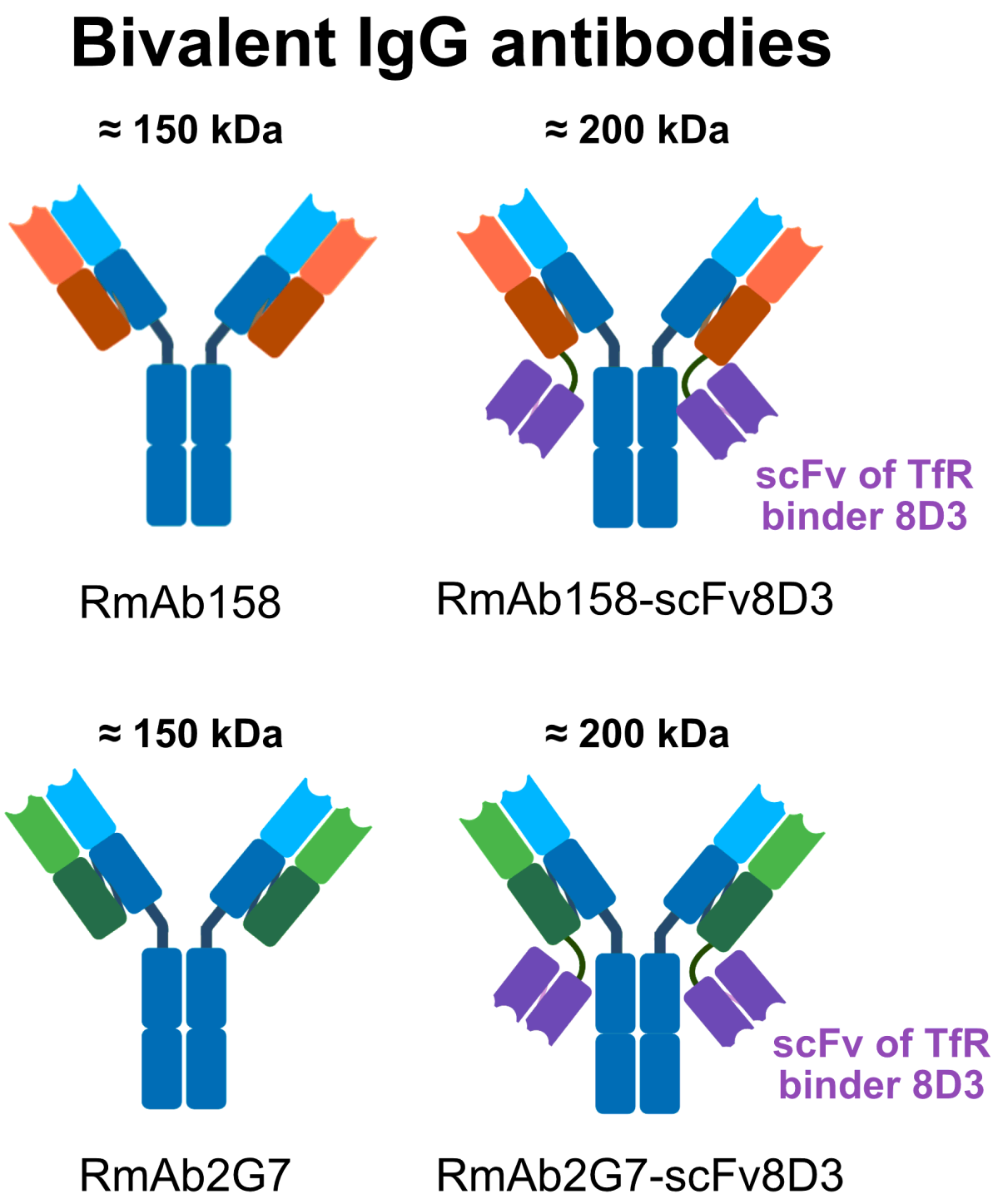

Figure 2

Cartoon representations of the two types of bivalent monoclonal antibody, with (RmAb158-scFv8D3 and RmAb2G7-scFv8D3) and without (RmAb158 and RmAb2G7) the transferrin receptor transporter scFv8D3, used for the In-Cell BBB-Trans assay characterization studies. Illustration created with Biorender.com.

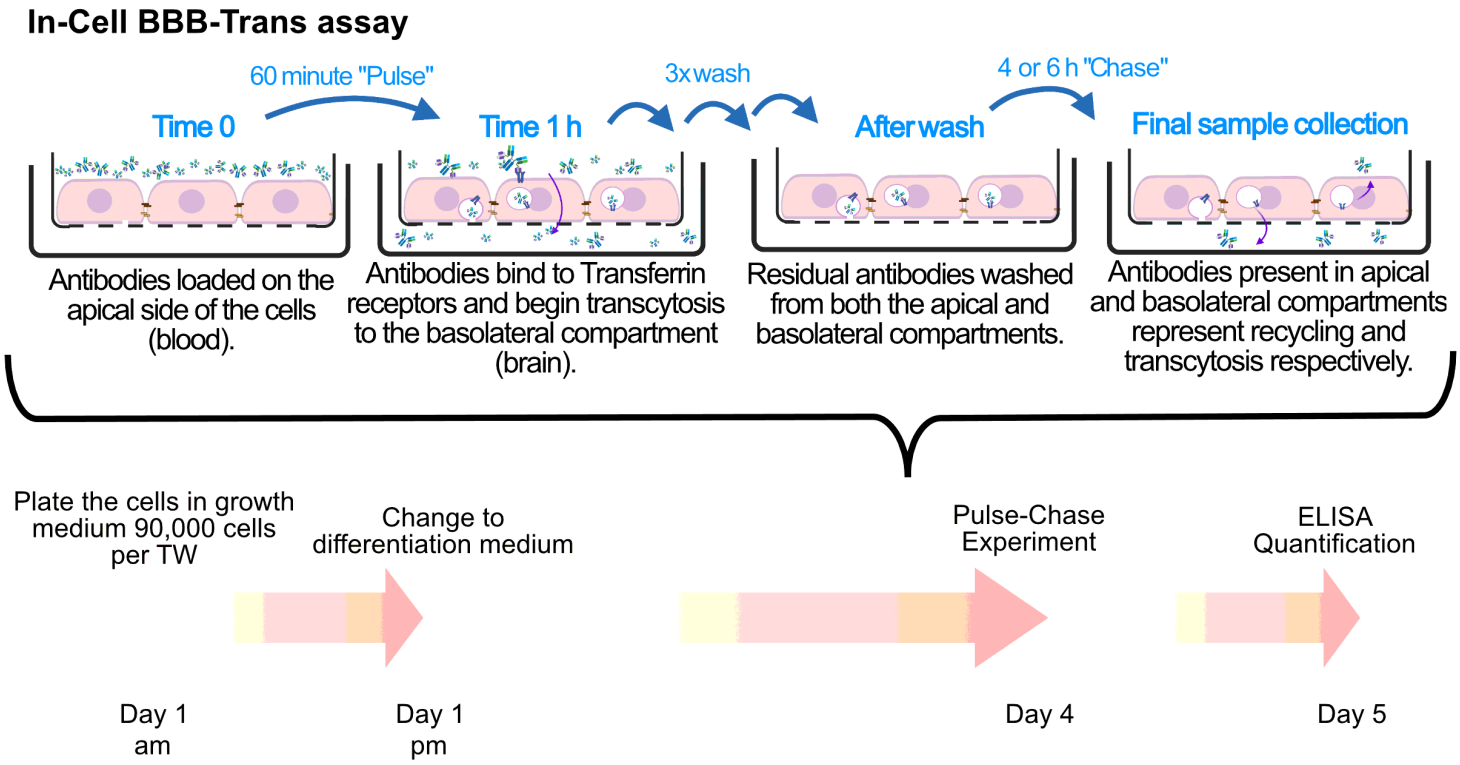

Figure 3

Diagram outlining the different phases of the In-Cell BBB-Trans assay setup, along with a proposed analytical time-line. Time 1h (*Pulse*), After Wash (*Wash*) and Final Sample Collection (*Chase*) are the three phases where media samples were taken for analysis. Illustration created with Biorender.com.

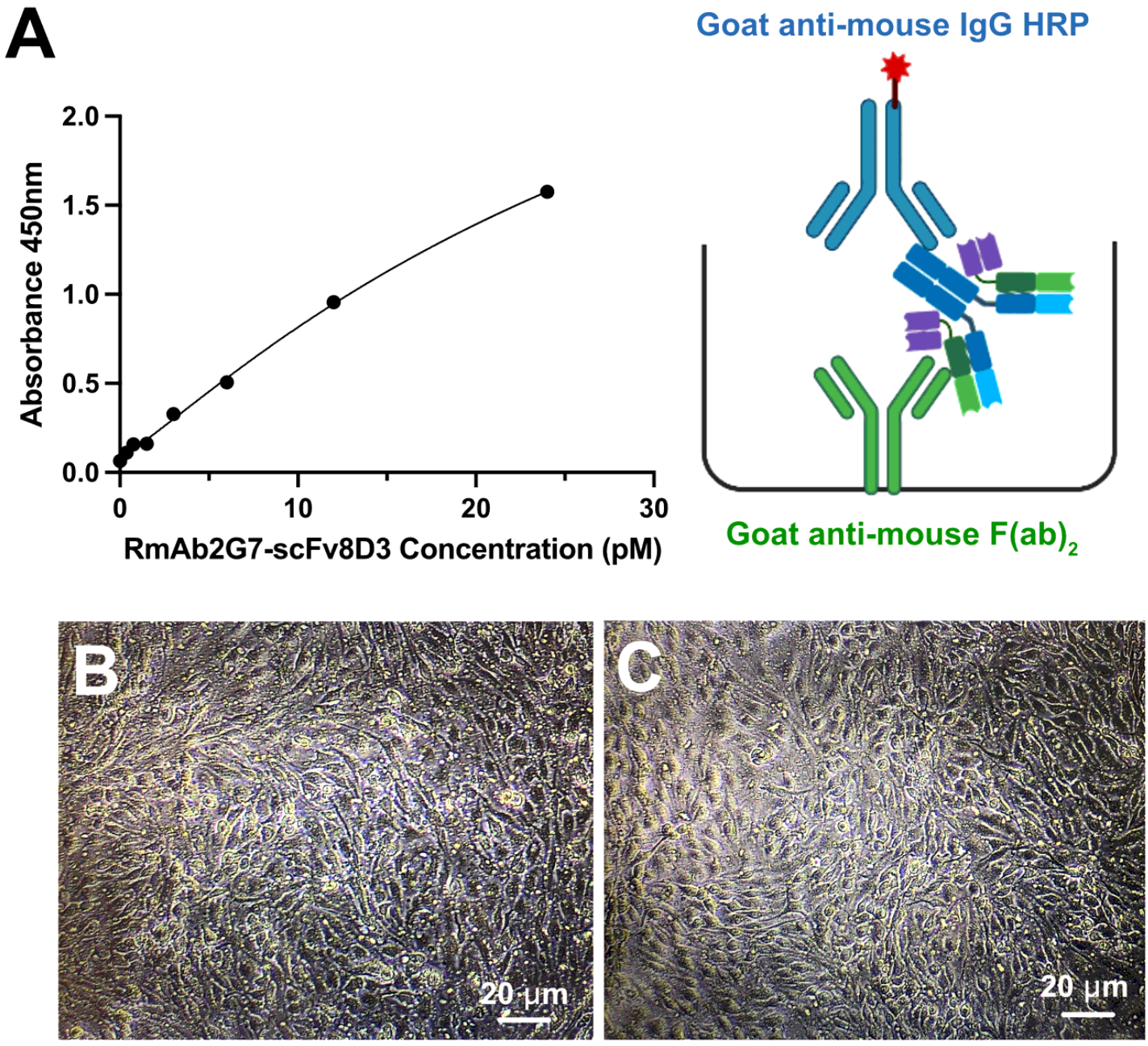

Figure 4

(A) Graphical representation of a routine ELISA standard curve for RmAb2G7-scFv8D3 obtained for interpolation of In-Cell BBB-Trans assay media samples, along with a cartoon representation of the sandwich ELISA setup to detect the pulsed monoclonal bivalent antibodies. Illustration depicting antibody binding in the ELISA created with Biorender.com. (B) and (C) Inverted light microscopic images of cEND cells grown on 0.4 mM pore transparent Bio-One® 24-well transwell membranes after three days in differentiation medium and following a 4-hour chase phase in serum-free medium respectively.

Figure 5

(A) Graphical representation of average antibody concentrations found in the apical and basolateral pulse compartments of cEND cell plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” with either 133.3 nM RmAb2G7 or RmAb2G7-scFv8D3 monoclonal bivalent antibodies. Six transwells were used for each pulsed antibody condition. The error bars represent 95 % confidence intervals. (B) Graphical representation of antibody concentrations found in the apical and basolateral pulse compartments of 0.4 mM translucent pore Bio-One® 24-well transwells, following a one-hour “pulse” of either 133.3 nM RmAb2G7 or RmAb2G7-scFv8D3 monoclonal bivalent antibodies. One transwell was used for each pulsed antibody condition.

Figure 6

(A) and (B) Photomicrograph representations of 8 mM sections of cEND cells grown on a 0.4 mM pore translucent Bio-One® 24-well transwell, immunofluorescently labelled with transferrin receptor (green), Rab5 (red) and DAPI (blue). The image in (A) was taken using an inverted epifluorescent microscope, with the dotted white lines demarcating the upper and lower boundaries of the transwell membrane. The image in (B) was taken using an inverted confocal microscope and processed using deconvoluting software. Scale bars were added to images (A) and (B) using ImageJ software.

Figure 7

(A) Graphical representation of average antibody concentrations found in the apical and basolateral 4-hour chase compartments cEND cell plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” with either 13.3 nM RmAb158 or RmAb158-scFv8D3 monoclonal bivalent antibodies. (B) Graphical representation of average antibody concentrations found in the apical and basolateral 4-hour chase compartments of cEND cell plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” with either 13.3 nM RmAb2G7 or RmAb2G7-scFv8D3 monoclonal bivalent antibodies. (C) Comparison of [125I] RmAb2G7 and [125I]RmAb2G7-scFv8D3 concentrations in the brain of 3-months old C57Bl/6 wild-type mice two and twenty-four hours post-injection. (D) Graphical representation of average antibody concentrations found in the apical and basolateral 6-hour chase compartments of cEND cell plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” with either 133.3 or 266.6 nM RmAb158 or RmAb158-scFv8D3 monoclonal bivalent antibodies. Six transwells were used for each pulsed antibody condition, except for 13.3 nM RmAb158 and RmAb158-scFv8D3 monoclonal bivalent antibodies (C), where five transwells were used. The error bars represent 95 % confidence intervals. ** Represents a significance level of $P<0.01$. *** represents a significance level of $P<0.001$.

Figure 8

(A) and (B) Cartoon representations of canonical endocytosis of the transferrin receptor bound monoclonal bivalent antibodies, conjugated to scFv8D3, in the absence or presence of the endocytosis inhibitor Dyngo4a™ respectively. Illustration created with Biorender.com. (C) Graphical representation of average antibody concentrations found in the apical and basolateral 4-hour chase compartments of cEND cell plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” of 13.3 nM RmAb2G7-scFv8D3 monoclonal bivalent antibody supplemented with DMSO (carrier) or 30 mM Dyngo4a™ 30 minutes “pre-pulse”, “pulse” and “chase” phases of the In-Cell BBB-Trans assay. Six transwells were used for each pulsed antibody condition. The error bars represent 95 % confidence intervals. ** represents a significance level of $P<0.01$.

Figure 9

Graphical representation of average antibody concentrations found in the apical and basolateral 4-hour chase compartments of Passage 11 and 48 cEND cells plated on 0.4 mM translucent pore Bio-One® 24-well transwell cultures, following a one-hour “pulse” of 13.3 nM RmAb2G7-scFv8D3. Six transwells were used for each pulsed antibody condition. The error bars represent 95 % confidence intervals. * represents a significance level of $P<0.05$. ** represents a significance level of $P<0.01$.

Supplementary Files

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- FigS1.png
- SupplementayFigure2.mov