

Amyloid-beta peptides 40 and 42 employ distinct molecular pathways for cell entry and intracellular transit at the BBB endothelium

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ABSTRACT

Blood-brain barrier (BBB) is a critical portal regulating the bidirectional transport of amyloid beta ($A\beta$) proteins between blood and brain. Disrupted trafficking at the BBB may not only promote the build-up of $A\beta$ plaques in the brain parenchyma, but also facilitate $A\beta$ accumulation within the BBB endothelium, which aggravates BBB dysfunction. Soluble $A\beta_{42}$: $A\beta_{40}$ ratios in plasma and cerebrospinal fluid have been reported to decrease during Alzheimer's disease (AD) progression. Our previous publications demonstrated that trafficking of $A\beta_{42}$ and $A\beta_{40}$ at the BBB is distinct and is disrupted under various pathophysiological conditions. However, the intracellular mechanisms that allow BBB endothelium to differentially handle $A\beta_{40}$ and $A\beta_{42}$ have not been clearly elucidated. In this study, we identified mechanisms of fluorescently labeled $A\beta$ (F- $A\beta$) endocytosis in polarized human cerebral microvascular endothelial (hCMEC/D3) cell monolayers using pharmacological inhibition and siRNA knock-down approaches. Further, intracellular transit of F- $A\beta$ following endocytosis was tracked using live cell imaging. Our studies demonstrated that both F- $A\beta$ peptides were internalized by BBB endothelial cells via energy, dynamin and actin dependent endocytosis. Interestingly, endocytosis of F- $A\beta_{40}$ is found to be clathrin-mediated, whereas F- $A\beta_{42}$ endocytosis is caveolae-mediated. Following endocytosis, both isoforms were sorted by the endo-lysosomal system. While $A\beta_{42}$ was shown to accumulate more in the lysosome which could lead to its higher degradation and/or aggregation at lower lysosomal pH, $A\beta_{40}$ demonstrated robust accumulation in recycling endosomes which may facilitate its transcytosis across the BBB. These results provide a mechanistic insight into the selective ability of BBB endothelium to transport $A\beta_{40}$ versus $A\beta_{42}$. This knowledge contributes to the understanding of molecular pathways underlying $A\beta$ accumulation in the BBB endothelium and associated cerebrovascular dysfunction as well as amyloid deposition in the brain parenchyma which are implicated in AD pathogenesis.

INTRODUCTION

Mounting evidence suggests that reduction in the ratio of amyloid beta peptide 42 (A β 42) and A β 40, two major A β isoforms that accumulate in AD patient plasma and the cerebrospinal fluid is associated with the severity of AD pathology and is emerging as a reliable biomarker for AD diagnosis¹⁻². Changes in A β 42/A β 40 ratio during AD progression may not be solely driven by the differential amyloidogenicity of A β 42 and A β 40; differential handling of A β 0 and A β 42 by the blood-brain barrier (BBB) and other disposition (distribution, metabolism, and elimination) pathways may also play a substantial role.

BBB is a physiological interface between blood and the brain which maintains the brain homeostasis by delivering essential nutrients to the brain and removing toxic metabolites out of the brain³⁻⁴. The carrier and barrier functions of the BBB are coordinated by selective internalization and intricate intracellular trafficking apparatus⁵. The luminal-to-abluminal transport of A β was reported to occur via the receptor for advanced glycation end products (RAGE)⁶, whereas the low-density lipoprotein receptor related protein-1 (LRP1)⁷⁻⁸ and P-glycoprotein (P-gp)⁹ were claimed to mediate brain-to-blood efflux. However, the molecular machinery by which these receptors/transporters mediate cell entry and subsequent intracellular transit of A β in the BBB endothelial cells is only partially characterized.

Impairment of A β transport at the BBB, is believed to promote the formation of cerebrovascular amyloid deposits and amyloid plaques found in the brains of Alzheimer's disease (AD) patients. Disrupted A β trafficking at the BBB is also presumed to increase anomalous accumulation of A β peptides in the BBB endothelium that compromise BBB integrity¹⁰ and function¹¹, and trigger inflammatory changes¹². Therefore, elucidating molecular mechanisms underlying A β trafficking disruption is critical for identifying novel therapeutic strategies.

Our recent study demonstrated that these two isoforms exhibit distinct trafficking and accumulation kinetics at the BBB endothelium¹³, which could be attributed to distinct trafficking

apparatus employed by A β 40 and A β 42, although they are thought to be transported by the same receptors/transporters. Elucidation of the underlying mechanisms by which A β isoforms are differentially internalized and sorted in the BBB endothelial cells may lead to the identification of molecular mediators that drive pathological shifts in A β 42/40 ratios in plasma and the brain during AD progression.

Extensive research describing the mechanisms underlying intraneuronal accumulation of A β peptides has been reported. Previous work conducted by us and others have demonstrated that A β 40 and A β 42 are internalized by distinct mechanisms¹⁴⁻¹⁵ and follow different itineraries in neuronal cells¹⁶. However, similar studies have not been conducted in BBB endothelial cells.

In the current study, we systematically dissected the cellular uptake and intracellular itineraries of fluorescently labeled soluble A β 40 and A β 42 (F-A β 40 or F-A β 42) in BBB cell culture models *in vitro*. We quantified the intracellular accumulation of F-A β using flow cytometry and confocal microscopy following pharmacological inhibition and siRNA knockdowns to investigate the contributions of known endocytic mechanisms. Further, we assessed the intracellular distributions of F-A β isoforms by labeling various endo-lysosomal organelles followed by live cell imaging. Our studies revealed that F-A β 40 and F-A β 42 are endocytosed by BBB endothelial cells via distinct molecular pathways and are differentially sorted by the endo-lysosomal system, which could lead to their distinct trafficking and accumulation kinetics in the BBB endothelium.

RESULTS

Energy dependent uptake of F-A β 40 and F-A β 42 in BBB endothelial cells. Receptor-mediated endocytosis requires energy produced by ATP hydrolysis. To determine if the uptake of F-A β 40 or F-A β 42 in human cerebral microvascular endothelial cell (hCMEC/D3) monolayers is mediated by energy-dependent endocytosis, the extent of F-A β internalization with or without ATP depletion was determined by flow cytometry (**Figure 1A**). As shown in the histograms, the intra-endothelial F-A β 40 (**Figure 1B**) and F-A β 42 (**Figure 1C**) accumulation was significantly reduced in ATP depleted cells compared to control cells. The corresponding geometric mean of fluorescence intensity and coefficient of variance were displayed in **Figure 1D**.

Dynamin dependent uptake of F-A β 40 and F-A β 42 in BBB endothelial cells. To determine if F-A β endocytosis is dependent on dynamin, a protein involved in pinching off the endocytotic vesicles¹⁷⁻¹⁸, the effect of dynasore, a potent dynamin inhibitor, on F-A β uptake was assessed by flow cytometry (**Figure 2A**). It was shown that uptake of F-A β 40 was significantly inhibited in dynasore pre-treated cells compared to the control cells (**Figure 2B**). Uptake of F-A β 42 also appeared to decrease in dynamin treated cells compared to the control cells; however, the difference was not statistically significant (**Figure 2C**). Effect of dynamin on F-A β internalization was further verified in hCMEC/D3 cells overexpressing dominant-negative mutant K44-dynamin (**Figure 2D**). Substantial decrease in green puncta of F-A β 40 and red puncta of AF633-Trf were observed in cells expressing K44-dynamin mutant (**Figure 2F**) compared to control cells (**Figure 2E**). Similarly, uptake of F-A β 42 was also inhibited in K44-dynamin transfected cells (**Figure 2H**) compared to control cells (**Figure 2G**). Moreover, cells that were transfected with GFP-K44 dynamin (green puncta, **Figure 2J**) clearly showed lower uptake of both SR101-A β 40 (orange puncta, **Figure 2K**) and AF633-Trf (red puncta, **Figure 2L**). However, in the same image, non-transfected cells without GFP expression demonstrated the uptake of both SR101-A β 40 and AF633-Trf.

Actin dependent uptake of F-A β 40 and F-A β 42 in BBB endothelial cells. To investigate the impact of actin on F-A β uptake. The intracellular accumulation of F-A β 40 and F-A β 42 in hCMEC/D3 cells was determined by flow cytometry with or without actin inhibition (**Figure 3A**). The histograms demonstrated that uptake of F-A β 40 (**Figure 3B**) and F-A β 42 (**Figure 3C**) was substantially reduced in cells pre-treated with cytochalasin A, which disrupts actin dynamics. Further, the reductions were found to be statistically significant for both F-A β 40 (**Figure 3D**) and F-A β 42 (**Figure 3E**) from three independent experiments.

F-A β 40 uptake by BBB endothelial cells via clathrin-mediated endocytosis. Flow cytometry analysis showed lower accumulation of F-A β 40 in cells treated with monodansylcadaverine (MDC) (**Figure 4A**) and chlorpromazine (CPZ) (**Figure 4C**) and the difference was found to be statistically significant (**Figure 4E**). Although, F-A β 42 uptake was reduced in MDC (**Figure 4B**) and CPZ (**Figure 4D**) pre-treated hCMEC/D3 cells, the difference was not significant (**Figure 4F**). To further confirm the role of clathrin-mediated endocytosis on F-A β uptake, knockdown of clathrin in hCMEC/D3 cells was performed using siRNA. Then the cells were incubated with AlexaFluor 633-transferrin (AF633-Trf) and F-A β (**Figure 4G**). The AF633-Trf (red puncta, **Figure 4H-K**) was used as a marker of clathrin-mediated endocytosis and its uptake was inhibited in the cells transfected with clathrin siRNA compared to the control cells that underwent mock transfection. In these cells, intracellular uptake of F-A β 40 was also significantly reduced compared to the control cells (green puncta, **Figure 4H-I**). However, no substantial change was observed in F-A β 42 uptake (green puncta, **Figure 4J-K**).

F-A β 42 uptake by BBB endothelial cells via caveolae-mediated endocytosis. Flow cytometry studies conducted on hCMEC/D3 cells demonstrated that nystatin (**Figure 5B**) or methyl- β -cyclodextrin (m β CD) (**Figure 5D**) treatment significantly reduced the F-A β 42 uptake (**Figure 5F**). However, no impact of nystatin on F-A β 40 uptake was observed as indicated by the histogram (**Figure 5A**) and statistical analysis (**Figure 5E**), although the fluorescence

intensity of F-A β 40 was still significantly reduced after m β CD treatment (**Figure 5C, E**). In a separate study, primary bovine brain microvascular endothelial (BBME) cells grown on Transwell® inserts were treated with m β CD and F-A β , followed by confocal microscopy imaging (**Figure 5G**). The z-stack images demonstrated reduced intracellular uptake of F-A β 42 (green puncta) in m β CD treated monolayers (**Figure 5K**) in comparison to control cells (**Figure 5J**). In contrast, the uptake of F-A β 40 was unaffected by m β CD pretreatment (**Figure 5H-I**).

Tyrosine phosphorylation of caveolin-1 is induced by A β 42 but not by A β 40 and

mediates A β 42 uptake. Caveolin-1 is a major structural component of caveolae, to further confirm the impact of caveolin-1 on cellular uptake of A β , knockdown of caveolin-1 in hCMEC/D3 cells was performed using siRNA and intracellular A β was measured by ELISA. Successful knockdown of caveolin-1 was demonstrated by western blotting as shown in **Figure 6A**. Moreover, caveolin-1 knockdown was shown to significantly reduce A β 42 cellular uptake whereas no significant difference was observed for A β 40 (**Figure 6B**). It has been reported that phosphorylation of caveolin-1 is associated with caveolae-dependent endocytosis¹⁹⁻²⁰. In order to study the effect of A β 40 and A β 42 on the phosphorylation of caveolin-1, we exposed hCMEC/D3 cells to soluble A β peptides predominantly containing monomers and determined the phospho-caveolin-1 expression by western blotting. Incubation with A β 42 resulted in a significant increase in the phosphorylation of caveolin-1 on tyrosine 14 and pretreatment of Src kinase inhibitor PP1 was shown to block the phosphorylation (**Figure 6C**). By contrast, A β 40 has no impact on caveolin-1 phosphorylation (**Figure 6D**).

Intracellular itineraries of A β 40 versus A β 42 in BBB endothelial cells.

Accumulation of A β 40 and A β 42 in early and late endosomes. hCMEC/D3 cells were transfected with m-cherry Rab5 protein (marker for early endosome) and then subjected to incubation with F-A β 40. Confocal microscopy studies demonstrated that A β 40 accumulated in the early endosomes as shown by substantial colocalization of F-A β 40 (**Figure 7B**) and m-

cherry Rab5 (**Figure 7A**). Further, F-A β 40 was found to accumulate in the late endosomes, as indicated by colocalization of F-A β 40 and Dil-LDL (**Figure 7D-F**). To confirm the accumulation of F-A β in the late endosomes, hCMEC/D3 cells transfected with GFP-labeled Rab7 protein (marker for late endosome) were incubated with SR-A β 40 or SR-A β 42 and then imaged live for up to 60 minutes. Only minor accumulation of SR-A β 40 in the late endosomes was observed at early time points (**Figure 8A-B**). Contrarily, substantial colocalization between SR-A β 42 and GFP-labeled Rab7 was found after 14 minutes of incubation (**Figure 8D-E**). Both peptides were shown to accumulate in the late endosome at later time points (**Figure 8C, F**).

Accumulation of A β 40 in recycling endosomes. Accumulation of A β 40 in recycling endosomes was examined in hCMEC/D3 cells transfected with GFP-labeled Rab11, which is a marker for recycling endosomes. Considerable colocalization of SR-A β 40 and GFP-labeled Rab11 was observed throughout the incubation period (**Figure 9**), confirming A β 40 accumulation in recycling endosomes.

Accumulation of A β 40 and A β 42 in lysosomes. hCMEC/D3 cells were co-incubated with F-A β and lysotracker (predominantly a marker for lysosomes) to examine lysosomal accumulation of F-A β . The F-A β 40 displayed only a partial localization in the lysosomes (**Figure 10A-D**), whereas F-A β 42 exhibited considerable lysosomal accumulation (**Figure 10E-H**). Pearson's correlation coefficient of F-A β 42, which describes its extent of colocalization with lysosomes was found to be significantly higher than that of F-A β 40 (**Figure 10I**).

DISCUSSION

BBB is a pivotal portal that regulates the bi-directional transport of A β mostly via receptor-mediated transcytosis, involving endocytosis, intracellular sorting, and exocytosis to the contralateral side²¹⁻²². Multiple receptors such as RAGE and LRP1 have been proposed to regulate influx and efflux of A β , respectively. Although both A β 40 and A β 42 were reported to be substrates of these receptors/transporters, these peptides exhibited distinct disposition profiles, and are differently impacted by various pathophysiological conditions. It has been previously reported that A β 40 is more vasculotropic than A β 42 and primarily accumulate as amyloid deposits in the brain blood vessels, whereas A β 42 forms the core of amyloid plaques that accumulate in the brain parenchyma²³. Moreover, the CSF levels of soluble A β 40 and A β 42 decrease with aging in AD transgenic mice²⁴ and AD patients². However, the magnitude of A β 42 decrease is greater than that of A β 40, thus resulting in a reduction in A β 42/A β 40 ratio. Therefore, A β 42/A β 40 ratio in CSF rather than A β 42 concentration alone is considered as a reliable biomarker to detect AD. Hence investigating molecular mechanisms that disrupt A β 42/A β 40 ratio during AD progression is expected to inform key pathophysiological pathways driving AD progression.

Although, several investigators opined that the reduction in soluble A β 42/A β 40 ratio is due to the propensity of A β 42 to form insoluble fibril and amyloid plaques, contribution of A β 42 and A β 40 trafficking disruptions at the BBB endothelium that could affect the levels of both isoforms in CSF and plasma cannot be ruled out. Bioinformatic analysis has shown that the genes encoding for various proteins involved in intracellular cargo sorting are downregulated in brains of AD patient brains²⁵. Therefore, we hypothesize that the BBB endothelium discriminates A β isoforms and traffics them independently via distinct transcytosis mechanisms. Selective disruption of transcytosis pathways that predominantly handle A β 40 versus A β 42 at the BBB may contribute to variations in A β 42/40 ratios observed during AD progression.

Moreover, dysregulated trafficking of A β peptides could promote their accumulation in the BBB endothelium and induce BBB dysfunction, which is associated with AD progression. However, the molecular mechanisms regulating intracellular trafficking and accumulation of A β in the BBB endothelial cells remain elusive. Therefore, in this study, we characterized the cellular mechanisms involved in A β internalization and intracellular transit at the BBB endothelium.

Our previous studies have shown that the uptake of A β 40 by bovine brain microvascular endothelial cells is temperature dependent¹⁴. Current study confirmed that the uptake of both A β 40 and A β 42 in hCMEC/D3 monolayers require energy and is inhibited upon ATP depletion (**Figure 1**). Further, our recent publication showed saturable uptake of A β by BBB endothelium²⁶. Taken together, these results demonstrated that both A β 40 and A β 42 are internalized by BBB endothelial cells via receptor-mediated endocytosis. Next, we have shown that A β internalization is dynamin dependent. Dynamin is recruited to the necks of vesicles formed during endocytosis and induces membrane scission to release vesicles into the cytosol¹⁷⁻¹⁸. By pretreating hCMEC/D3 monolayers with dynasore (dynamin inhibitor) followed by flow cytometry and transfection of mutant form of dynamin followed by confocal microscopy, we demonstrated that dynamin is involved in the endocytosis of both F-A β 40 and F-A β 40 (**Figure 2**). Previous studies have shown that the uptake of A β 42 but not A β 40 is reduced in dynasore treated PC-12 cells¹⁶ and SH-SY5Y cells¹⁵. The discrepancy between BBB endothelial cells and neuronal cells could be due to differential expression of dynamin isoforms. While dynamin 1 and 3 are highly expressed in neurons, endothelial cells are believed to mostly express dynamin 2²⁷.

During endocytosis, actin polymerization provides the force for vesicle budding and has been reported to mediate uptake of cargos in various cells²⁸⁻²⁹. In the current study, we found that both A β 40 and A β 42 utilize actin-dependent endocytic mechanisms to enter the BBB endothelial cells (**Figure 3**). Similar results were reported in neurons¹⁵ and astrocytes³⁰, where disruption of actin polymerization by cytochalasin D was shown to significantly reduce A β 40 and

A β 42 endocytosis. These studies have indicated that both A β 40 and A β 42 are internalized by BBB endothelial cells via energy, dynamin and actin-dependent endocytosis.

We further investigated specific mechanisms involved in A β endocytosis. Clathrin and caveolae-mediated endocytosis are the two well-studied mechanisms and were shown to facilitate the receptor-mediated transcytosis (RMT) of various macromolecules at the BBB. Endocytosis of critical endogenous proteins such as transferrin³¹, LDL³², and insulin³³ is mediated by clathrin. In contrast, caveolae-mediated transcytosis mediates the blood-to-brain entry of viruses and extracellular proteins such as albumin³⁴. Dysregulated caveolae-mediated transcytosis is believed to increase BBB permeability under pathological conditions triggered by ischemic stroke³⁵ and sub-arachnoid hemorrhage³⁶ or under exposure to low-intensity focused ultrasound³⁷. Therefore, we investigated the involvement of both clathrin and caveolae mediated endocytic mechanisms in A β uptake at the BBB endothelium. Using chemical inhibitors that inhibit the clathrin-coated pit assembly at the plasma membranes, MDC and CPZ³⁸⁻³⁹, we have shown that the endocytosis of A β 40 but not A β 42 is clathrin-mediated in hCMEC/D3 monolayers (**Figure 4A-F**). These results were further verified by confocal microscopy which demonstrated significant reduction in the uptake of F-A β 40 but not F-A β 42 by hCMEC/D3 monolayers in which clathrin was knockdown using siRNA (**Figure 4H-K**). It has been reported that clathrin-mediated endocytosis pathway is disrupted in AD⁴⁰⁻⁴², which is also reflected in impaired A β 40 endocytosis at the BBB. Indeed, Zhao et. al have found that PICALM regulates clathrin-dependent internalization of A β bound to LRP1, leading to BBB-mediated clearance of A β . Further, PICALM levels and A β clearance were shown to be reduced in AD-derived endothelial monolayers⁴³. On the other hand, inhibitors of caveolae-mediated endocytosis were shown to significantly decrease the uptake of A β 42 but not A β 40 as shown by confocal microscopy (**Figure 5H-K**). Although m β CD also significantly reduced the A β 40 uptake in flow cytometry analysis, this inhibitor is non-specific and acute cholesterol depletion by m β CD has also been

reported to inhibit clathrin-mediated endocytosis⁴⁴. Involvement of caveolae-mediated endocytosis in A β 42 uptake was further confirmed by siRNA knockdown studies showing that caveolin-1 knockdown significantly reduced A β 42 uptake but had no impact on A β 40. Moreover, western blot results demonstrated that only A β 42, not A β 40, increased the tyrosine-14 phosphorylation of caveolin-1 (**Figure 6**). Src-dependent caveolin-1 phosphorylation is suggested to facilitate the formation and internalization of caveolae into the cytoplasm^{19, 45-46}; phosphorylation associated conformational change of caveolin-1 is believed to engender maturation of caveolae and subsequent release from the plasma membrane²⁰. Together, these studies indicate that the endocytosis of A β 40 at the BBB endothelium is primarily clathrin-dependent whereas the endocytosis of A β 42 is caveolae-mediated.

Differential involvement of endocytic pathways between A β 40 and A β 42 could provide mechanistic foundations underlying differential transport kinetics of these two peptides across the BBB in various pathophysiological conditions. For example, our previous study illustrated that peripheral insulin exposure decreased the brain influx of A β 42 but increased A β 40 influx. Further, insulin treatment in vitro enhanced the uptake of transferrin, a clathrin-mediated endocytosis marker, by BBB endothelial cells but reduced caveolae-mediated endocytosis of AF647-cholera toxin-B⁴⁷. Hence, the differential effects of insulin on A β trafficking could be due to distinct endocytic pathways employed by these two peptides, which allows insulin to activate one while inhibiting the other. The molecular mechanism by which insulin exerts such selective effect on clathrin and caveolae-mediated endocytosis is not fully understood and the role of insulin signaling pathway in mediating these processes is currently being investigated in our lab. In another study, we demonstrated that blood-to-brain influx of A β 40 decreased whereas A β 42 increased with aging in WT mice⁴⁸. Age-related shift from ligand-specific transport to non-specific caveolar transcytosis at the BBB has been previously reported⁴⁹. Given the current finding that A β 42 endocytosis at the BBB endothelium is caveolae-mediated, preferential influx

of A β 42 during aging is most likely due to the shifts in transcytosis that involves clathrin versus caveolae-coated vesicles.

Following the receptor-mediated endocytosis, cargos are sorted via the endo-lysosomal system constituting of early, late, recycling endosomes and lysosomes. In polarized BBB endothelial monolayers, such sorting system determines the fate of lysosomal degradation, recycling back to the plasma membrane, or transcytosis⁵⁰⁻⁵². Therefore, in the second part of this study, we investigated the intracellular itineraries of A β 40 and A β 42 in polarized hCMEC/D3 cell monolayers. We found that the majority of internalized A β 40 trafficked through the early and late endosomes as assessed by the co-localization of F-A β 40 with m-cherry-Rab5 and Dil-LDL (**Figure 7**). Accumulation of A β 40 and A β 42 in the early endosomes was also reported in differentiated PC12 cells¹⁶ and mouse neuroblastoma N2a cells⁵³. From the early endosome, A β is recycled to the plasma membrane or emptied into late endosomes and lysosomes for degradation. Live cell imaging has shown that SR-A β 40 accumulation in late endosomes occurred with longer incubation times (40-60 minutes). In contrast, SR-A β 42 rapidly entered late endosomes with shorter as well as longer periods of incubation (**Figure 8**). Subsequently, greater accumulation of F-A β 42 was observed in the lysosomal compartment compared to F-A β 40 (**Figure 10**). These results were consistent with our previous findings in neuronal cells¹⁶ and implies that A β 42 is more susceptible to lysosomal degradation compared to F-A β 40. On the other hand, F-A β 40 accumulated in GFP-Rab11 labeled recycling endosomes up to 1 hour of incubation (**Figure 9**). The Rab11 has been reported to regulate exocytosis of recycling vesicles at the plasma membrane and a recent study suggested that it also mediates the transcytosis of polymeric immunoglobulin A (pIgA) across the polarized epithelial cells⁵⁴⁻⁵⁵. Our previous study also demonstrated that the exocytosis rate of intracellular A β 40 to the abluminal side (transcytosis) was significantly higher than that to the luminal side (recycling)⁴⁷. These

results indicate that internalized A β 40 and A β 42 follow distinct trafficking paths in the BBB endothelium, which may determine their transcytosis potential versus intracellular degradation.

Published studies indicated that caveolae-mediated vesicle trafficking directs monocarboxylic acid transporter 1 (Mct1) into late endosome/lysosome compartments in BBB endothelial cells, whereas clathrin-mediated endocytosis directs Mct1 to Rab11-positive recycling endosomes⁵⁶. Therefore, caveolae-mediated endocytosis could sort A β 42 for lysosomal degradation while clathrin-mediated endocytosis moves A β 40 into recycling endosomes. Another potential reason for the selectivity between lysosomes and recycling endosomes could be related to their binding affinity to the receptors. Transferrin receptor antibodies are being employed as Trojan horses for delivering cargo across the BBB. Preclinical studies have shown that the transcytosis efficiency of this antibody is influenced by its affinity to the transferrin receptor. For instance, Johnsen et al. found that antibodies with low affinities mediated higher uptake of gold nanoparticles into the brain than the antibodies with higher affinities⁵⁷. Both in vivo pharmacokinetic modeling¹³ and cellular transcytosis²⁶ studies in our previous publications demonstrated that A β 42 has lower Michaelis constant (Km) than A β 40, suggesting higher binding affinity of A β 42 to the BBB endothelium. Like observed with transferrin receptor antibody, it is likely that such high binding affinity reduces A β 42 transcytosis and increases its likelihood of lysosomal entrapment.

Extensive accumulation of A β 42 (from 25 nM in extracellular fluid to 2.5 μ M in the lysosomal compartment) in the acidic environment of lysosomes could trigger its misfolding and enhance the formation of insoluble aggregates⁵⁸. The misfolded A β 42 aggregates were shown to disrupt the integrity of endosomal-lysosomal system and decrease the uptake of ovalbumin in primary neurons⁵⁹. It is possible that the accumulation of A β 42 in the lysosomes of BBB endothelial cells could have similar detrimental effects and cause disrupted trafficking of various cargos via the BBB. Moreover, A β 42 oligomers were found to damage tight junctional proteins

and result in paracellular leakage via activation of autophagy and up-regulation of metalloproteinases in murine brain capillary endothelial cells (bEnd.3)^{10, 60}.

Taken together, our study demonstrated that A β 40 and A β 42 are internalized by the BBB endothelial cells via dynamin and actin-dependent endocytosis but employ different endocytotic pathways. Endocytosis of A β 40 was found to be clathrin-mediated whereas A β 42 endocytosis is caveolae-mediated. Following endocytosis, both isoforms were sorted by the endo-lysosomal system; however, A β 42 was shown to accumulate more in lysosomes than A β 40, which could lead to higher degradation and/or aggregation of A β 42. These findings were summarized in **Figure 11**. Our results provided molecular insights into mechanisms that regulate the transport A β 40 versus A β 42 in the BBB endothelium. This knowledge is critical to understand mechanisms underlying A β accumulation in the BBB endothelium and resultant BBB dysfunction, which is widely believed to aggravate AD progression.

MATERIALS AND METHODS

Cell culture. Human brain endothelial (hCMEC/D3) cells were grown according to the culture conditions reported previously⁶¹. Polarized hCMEC/D3 cell monolayers were cultured on collagen (Corning, MA) coated coverslip bottomed dishes (Mattek, MA), on 6 well plates, or on Transwell[®] filters (Corning Costar[™], MA) at 5 % CO₂ and 37 °C. All the monolayers were moved to low-serum (1 % serum) medium, one night before the experiment. Bovine brain microvascular endothelial (BBME) cells were obtained from Cell Applications Inc. (San Diego, CA). The BBME cellular monolayers were grown as reported previously⁶². Formation of monolayers on Transwell[®] filters with good tight junctional integrity was confirmed by measuring the trans-endothelial electrical resistance (TEER) across the monolayers.

Microscopy and cellular imaging. The hCMEC/D3 or BBME monolayers were treated with 1 μM of F-Aβ₄₀, F-Aβ₄₂, Sulforhodamine101 (SR101) labeled Aβ₄₀, or SR101-Aβ₄₂ for 60 min followed by 20 μg/mL of AlexaFluor labeled transferrin (AF633-TRF) treatment for 30 min. In case of inhibitor experiments, the cells were pretreated with inhibitor for the designated time and then incubated with F-Aβ. Then the cells were washed with PBS and the nuclei were stained by incubating with Hoechst dye (0.5 μg/mL in PBS) for 5 min. At the end, the dishes were fixed in 4 % para-formaldehyde (PFA) at 4 °C for 60 min; mounted with ProLong[®] gold antifade reagent (Life technologies, OR); and dried overnight before imaging by laser confocal microscopy as described in our previous publication¹⁴.

Clathrin/caveolin-1 knockdowns and K44-negative dominant dynamin transfections.

Clathrin and caveolin-1 knockdown in polarized hCMEC/D3 cell monolayers was performed using siRNA kit containing RNAi mix (Invitrogen, CA) and reduced serum medium, Opti-MEM[™] (Gibco, NY). The cells were allowed to recover for 48 h in hCMEC/D3 medium (5 % FBS) before the uptake experiment were performed. GFP and non-GFP K44-negative-dominant dynamin transfections of hCMEC/D3 cell monolayers were performed using Lipofectamine reagent

(Invitrogen, CA) as per manufacturer's protocol. The cells were allowed to recover for 24 h in hCMEC/D3 medium (1 % FBS) and the experiment was performed the next day.

Rab protein transfections. m-Cherry fluorescent protein fused to Rab5 (m-CFP/Rab5), green fluorescent protein fused to Rab7 (GFP/Rab7) and green fluorescent protein fused to Rab11 (GFP/Rab11) plasmids were obtained as described in our previous publications²⁵. Transfections of hCMEC/D3 cell monolayers were performed using Lipofectamine reagent (Invitrogen, CA) as per manufacturer's protocol. The cells were allowed to recover for 24 h in hCMEC/D3 medium (1 % FBS) and the experiment was performed the following day.

Flow cytometry. Following the treatment with 1 μ M F-A β for 60 minutes, hCMEC/D3 cells were washed thoroughly with PBS and gently trypsinized with trypsin-EDTA for 2 minutes and neutralized with FBS. In case of inhibitor experiments, the cells were pretreated with inhibitor for the designated time and then incubated with F-A β . The dislodged cells were washed twice using ice cold PBS, fixed with 4 % PFA solution and analyzed for intracellular fluorescence using BD FACSCalibur™ flow cytometer. The F-A β 40 and F-A β 42 intracellular fluorescence intensities were measured using 488 nm laser fitted with 530/30 filter. Data was acquired with BD CellQuest™ Pro and analyzed using FlowJo software. The F-A β uptake was represented as histograms of intracellular fluorescence intensities. All sample analyses were performed within one hour from the completion of the experiment.

Western blotting. Following the treatment with 0.25 μ M A β 40 or A β 42 in DMEM for 15 minutes, the cells were washed three times with PBS and lysed with a RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Total protein concentration in the lysates was determined by bicinchoninic acid (BCA) assay (Pierce, Waltham, MA). Lysates (25 μ g protein per lane) were loaded onto 4-12% Criterion XT precast gels and proteins were separated by SDS-PAGE under reducing conditions (Bio-Rad Laboratories, Hercules, CA). The proteins were then electroblotted onto a 0.45 μ m nitrocellulose membrane. Membranes were

blocked with 5% nonfat dry milk protein (Bio-Rad Laboratories, Hercules, CA), followed by overnight incubation at 4 °C with primary antibodies (1:1000) against: phospho-caveolin-1 (Y14) (#3251), caveolin-1 (#3267), Vinculin (#13901) (Cell Signaling Technology, Danvers, MA). Afterwards, the membrane was incubated with IR-dye conjugated secondary antibody (1:2000) for 1 h at room temperature. Immuno-reactive bands were then imaged (Odyssey CLx; LI-COR Inc, Lincoln, NE) and the band intensities were quantified by densitometry (Image Studio™ Lite Software, LI-COR Inc, Lincoln, NE).

ELISA. Following the 48-hour recovery of vehicle/siRNA transfections, hCMEC/D3 cells were incubated with 1 μ M A β 40/42 for 30 minutes. Afterwards, cells were washed thoroughly with ice-cold PBS twice and harvested in 0.25% trypsin-EDTA. The resulting cell pellets were lysed in 50 μ L RIPA buffer containing protease inhibitor. The cell lysates were centrifuged at 10,000 rpm for 10 minutes to remove cell debris and used as samples for ELISA. A β levels were determined with A β 40- and A β 42-specific ELISA kits ([KHB3481](#) and KHB3544, ThermoFisher) according to the manufacturer's instructions. For data analysis, A β concentration was normalized by the total protein concentration and fold change compared to untreated control was calculated. The same cells lysates were also subjected to western blotting as described above in order to confirm the successful knockdown of caveolin-1.

Live cell imaging. Following the incubation with various fluorophores the cells were washed twice, maintained in an atmosphere humidified with 5% CO₂ in air, and imaged live using a TE-2000-S inverted microscope (Chiyoda-ku, Tokyo 100-8331, Japan) equipped with Nikon FITC HQ and m-Cherry-A-zero filters. The images were captured using Nikon's NIS elements AR 3.0 software and processed using ZEN Imaging software.

Mechanisms of F-A β 40 and F-A β 42 endocytosis at the BBB endothelium.

1. *Energy dependence of F-A β uptake.* The polarized hCMEC/D3 cell monolayers grown on Transwell® filters and 6-well culture plates were pre-incubated with glucose free DMEM supplemented with 50 mM 2-deoxy glucose and 0.1 % sodium azide (ATP depletion medium) for 30 min before start of the experiment. The control cells were incubated with regular DMEM medium containing glucose. Then, 1 μ M of either F-A β 40 or F-A β 42 was added to the luminal side of the Transwell® filter and incubated for 30 min. The resultant intracellular fluorescence was assessed by flow cytometry.

2. *Dynamin, actin, clathrin and lipid raft mediated F-A β endocytosis.* Small molecule inhibitors were used to inhibit the dynamin dependent vesicle pinch-off, actin polymerization, clathrin-dependent endocytosis, or lipid raft-mediated uptake to assess the contribution of these processes to the internalization of F-A β 40 and F-A β 42 by the BBB endothelium. In these experiments, polarized hCMEC/D3 monolayers were pre-incubated with medium containing 1 % serum for at least one hour before the addition of inhibitors. The hCMEC/D3 cells grown on 6-well culture plates or on Transwell® filters were pre-incubated with inhibitors for 30 min, then 1 μ M of F-A β 40 or F-A β 42 was added and the monolayers were incubated for 60 min. The uptake of F-A β peptides was determined using flow cytometry. The following inhibitors and concentrations were used: 80 μ M Dynasore (Tocris bioscience, MO) (dynamin inhibitor), 10 μ M cytochlasin A (Cayman Chemical, MI) (actin inhibitor), 200 μ M monodansylcadaverine (MDC, specific clathrin inhibitor), 10 mM chlorpromazine (MP Biomaterials, OH) (CPZ, non-specific clathrin inhibitor), 2.5 μ M nystatin (specific lipid raft inhibitor), 5 mM methyl- β -cyclodextrin (Acros Organics, NJ) (m β CD, non-specific cholesterol chelator). All the control experiments were performed in a similar fashion without the addition of inhibitors and the intracellular fluorescence was assessed by flow cytometry. For knockdown studies, control or knock-down cells were treated with F-A β peptides and AlexaFluor 633-transferrin (AF633-Trf) for 60 minutes. The

confocal images were obtained after washing the cells with PBS and fixing with 4 % PFA for 1 h at 4 °C.

In a separate experiment, polarized BBME cell monolayers were also pre-incubated with 10 mM m β CD for 30 min, followed by incubation with 1 μ M of F-A β 40 or F-A β 42, and 20 μ g/mL Alexafluor[®] labeled Transferrin (AF633-TRF) for 60 min. The z-series confocal micrographs were obtained after washing the filters with PBS and fixing with 4 % PFA for 1 h at 4 °C. Then Transwells[®] were mounted with Vectashield[®] Antifade mounting medium with DAPI (Vector Laboratories, CA).

Intracellular itineraries of F-A β 40 and F-A β 42 at the BBB endothelium.

1. *Accumulation in Early Endosomes.* hCMEC/D3 cells expressing m-CFP/ Rab5 were incubated with 1 μ M F-A β 40 for 15 minutes. Live cell imaging was conducted up to 60 minutes after washing the cells with DMEM.
2. *Accumulation in Secondary Endosomes.* The cells were treated with 1 μ M F-A β 40 and Dil-LDL (15 μ g/ml) for 60 minutes. Then live cell imaging was conducted after washing the cells with DMEM.
3. *Accumulation in Late Endosomes.* hCMEC/D3 cells expressing GFP labeled Rab7 were incubated with 1 μ M SR101-A β 40 or SR101-A β 42 for 15 minutes. Then the cells were washed with DMEM and imaged by live cell imaging up to 60 minutes.
4. *Accumulation in Recycling Endosomes.* hCMEC/D3 cells expressing GFP/Rab11 were incubated with 1 μ M SR101-A β 40 or SR101-A β 42 for 15 minutes. Then the cells were washed with DMEM and imaged by live cell imaging up to 60 minutes.

5. *Accumulation in Lysosomes.* The cells grown on coverslip-bottom dishes were treated with 1 μ M F-A β 40 or F-A β 42 and 75 nM LysoTracker Red[®] (Invitrogen-Molecular Probes, Carlsbad, CA) for 30 min at 37 °C. Thereafter, the cells were washed with ice-cold PBS 3 times and imaged by confocal microscopy. The fluorescein and LysoTracker Red signals in the image were superimposed, and the extent of colocalization was estimated by Pearson's correlation coefficients.

FIGURES

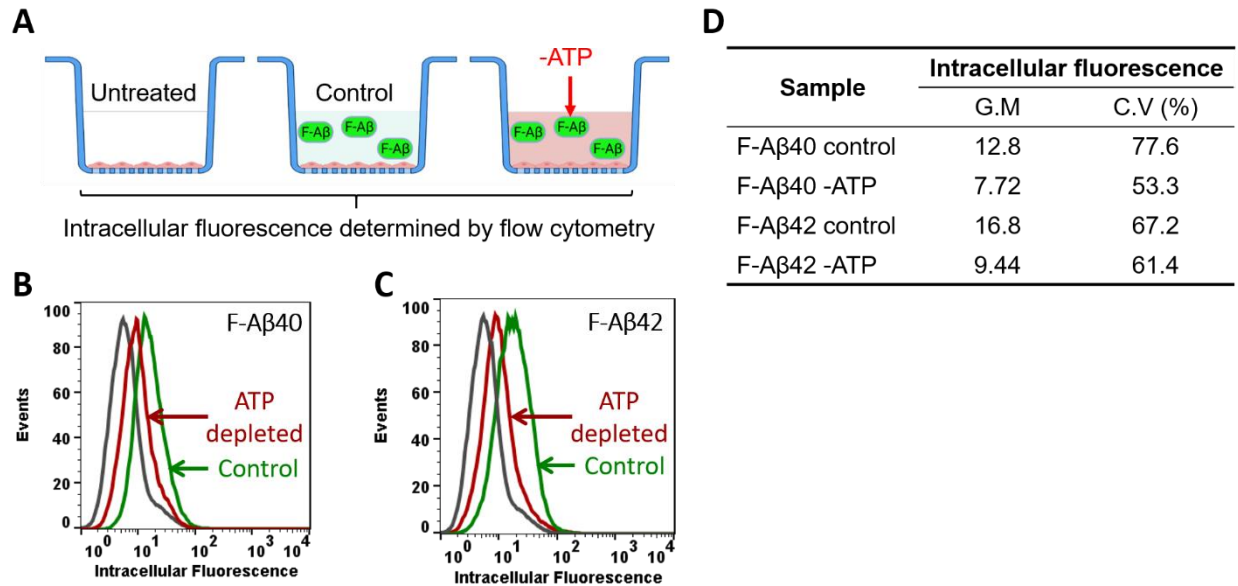


Figure 1. Energy dependent uptake of F-A β peptides in polarized hCMEC/D3 monolayers.

(A) Experimental design. Cells grown on Transwell[®] filters were pre-incubated with glucose free DMEM supplemented with 50mM 2-deoxy glucose and 0.1% sodium azide for 30 min. Then 1 μ M F-A β was added to the luminal compartment and incubated for 30 min. Intracellular fluorescence was assessed by flow cytometry. **(B-C)** Histograms of hCMEC/D3 cells incubated with **(B)** F-A β 40 or **(C)** F-A β 42 in control and ATP depleted group. **(D)** Comparison of geometric means of F-A β 40 and F-A β 42 fluorescence in control and ATP depleted cells.

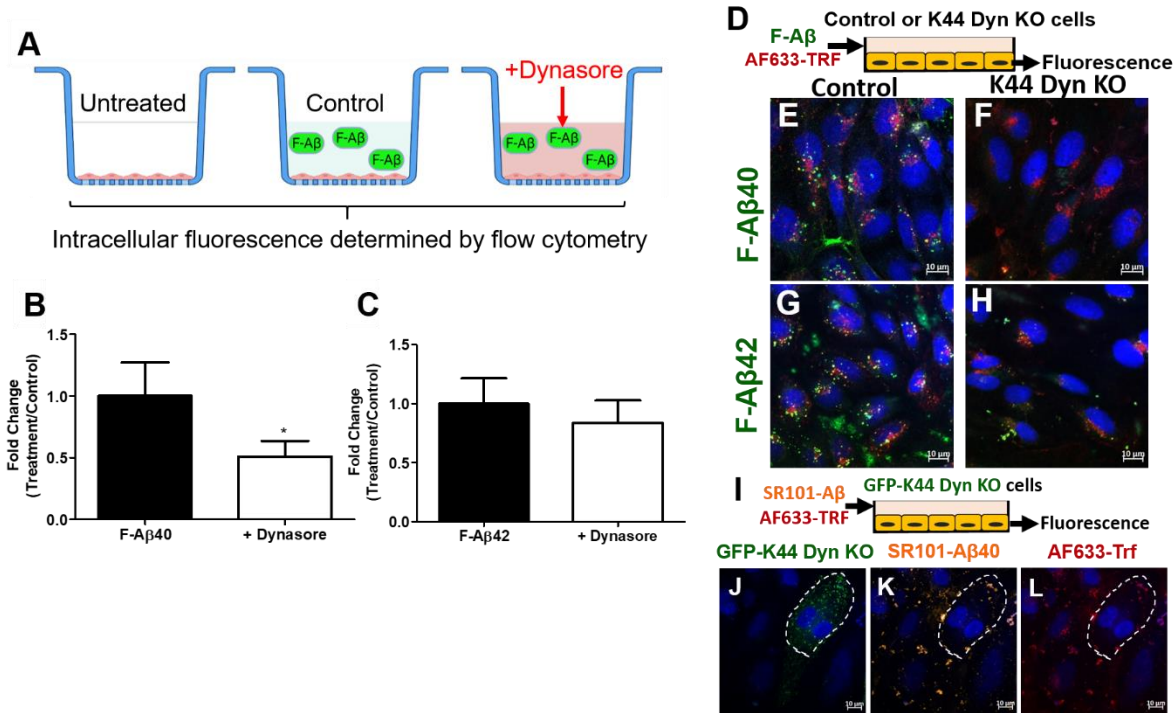


Figure 2. Dynamin dependent endocytosis of F-Aβ40 and F-Aβ42 in hCMEC/D3 cells. (A)

Experiment design of flow cytometry study. Cells grown on Transwell® filters were pre-incubated with 80 μM dynasore for 30 min. Then 1 μM F-Aβ was added to the luminal compartment and incubated for 60 min. Intracellular fluorescence was assessed by flow cytometry. **(B-C)** Fold change of median cellular fluorescence intensity from three replicates (n=3). *p<0.05, student's t-test. **(D, I)** Experiment design of confocal microscopy. Cells grown on coverslip bottom dishes were transfected with GFP or non-GFP K44-negative-dominant dynamin. Transfected cells were incubated with 1 μM F-Aβ or Sulforhodamine101 labeled Aβ (SR- Aβ) for 60 min followed by 20 μg/ml of AlexaFluor labeled transferrin (AF633-TRF) treatment for 30 min. Following fixation and nuclear staining, the cells were imaged by laser confocal microscopy. **(E-H)** Confocal micrographs depicting internalization of F-Aβ (green) and AF633-Trf (red) in **(E, G)** control cells and in **(F, H)** cells expressing negative-dominant K44-dynamin mutant (K44 Dyn KO). **(J-L)** Intracellular uptake of AF633-Trf (red) and SR-Aβ40, orange) in cells expressing GFP labeled negative-dominant K44-Dynamin (GFP-K44 Dyn KO). **(J)** GFP-K44 Dyn KO cells marked by dashed white line, **(K)** SR-Aβ40, and **(L)** AF633-Trf.

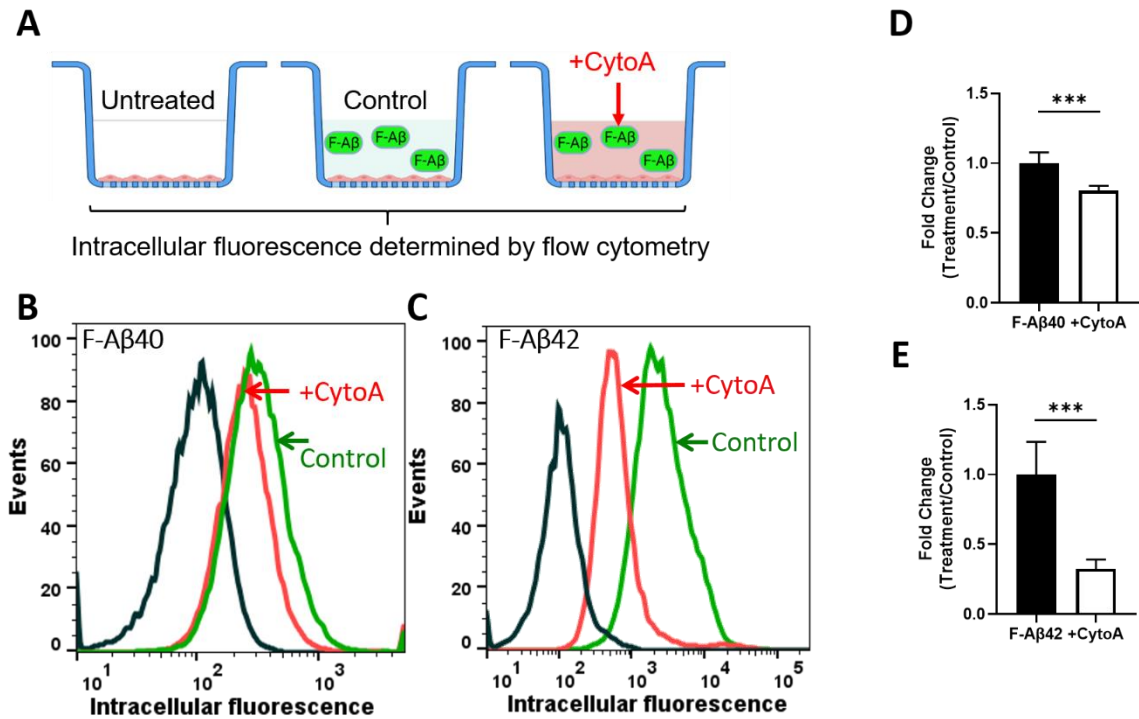


Figure 3. Actin dependent endocytosis of F-Aβ peptides in polarized hCMEC/D3

monolayers. (A) Experimental design. Cells were pre-incubated with cytochalasin A (CytoA, 10 μM) for 30 min. Then 1 μM F-Aβ F-Aβ was added and incubated for 1 hour. Intracellular fluorescence was assessed by flow cytometry. (B-C) Histograms of hCMEC/D3 cells incubated with (B) F-Aβ40 or (C) F-Aβ42 in control and CytoA treated group. (D-E) Fold change of median cellular fluorescence intensity of the total number of gated cells for three replicate samples (n=3). ***p<0.001, student's t-test.

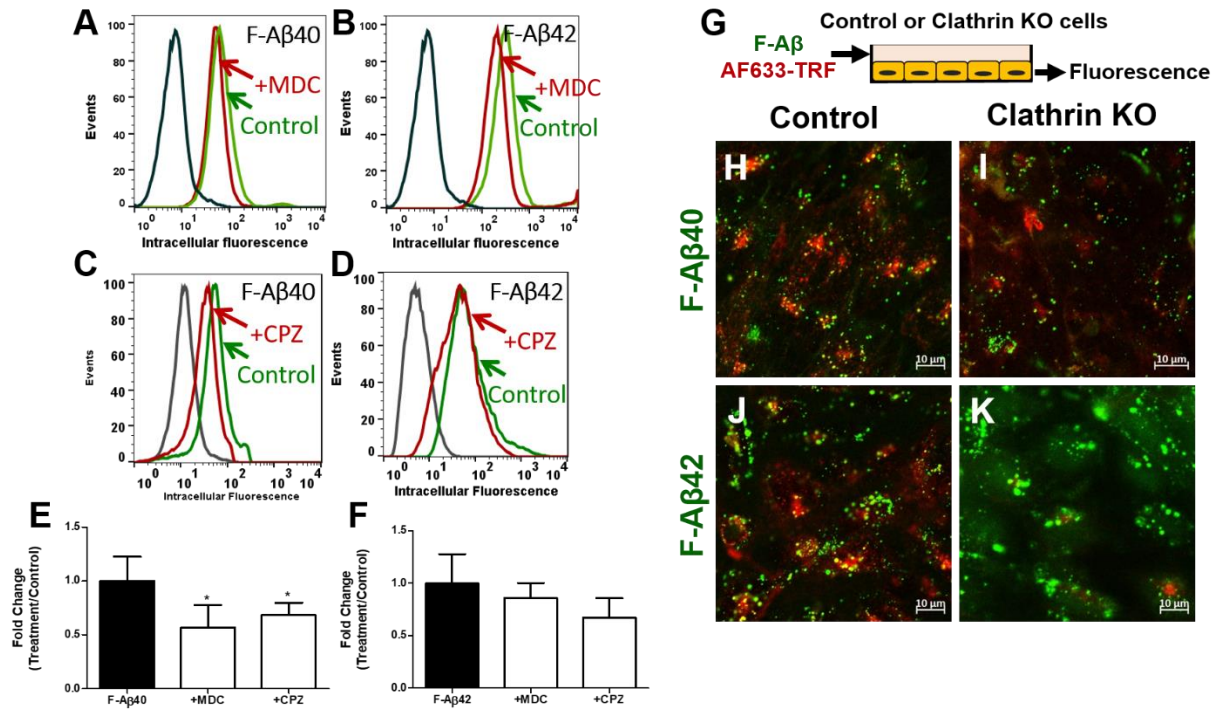


Figure 4. Role of clathrin-mediated endocytosis in the internalization of F-Aβ40 and F-Aβ42 in polarized hCMEC/D3 monolayers. (A-D) Histograms of hCMEC/D3 cells incubated with **(A)** F-Aβ40 and **(B)** F-Aβ42 in control and monodancyl cadverin (MDC, a specific clathrin inhibitor) pretreated group, **(C)** F-Aβ40 and **(D)** F-Aβ42 in control and chlorpromazine (CPZ, a non-specific clathrin inhibitor) pretreated group. **(E-F)** Fold change of median cellular fluorescence intensity from three replicates (n=3). *p<0.05 compared to F-Aβ alone group, one-way ANOVA with Bonferroni post-tests. **(G)** Experiment design of confocal microscopy study. Control or clathrin knocked-down cells were treated with 1 μM F-Aβ peptides and 20 μg/mL AlexaFluor 633-transferrin (AF633-Trf) and then subjected to confocal microscopy. **(H-K)** Confocal micrographs depicting internalization of F-Aβ (green) and AF633-Trf (red) in **(H, J)** control cells and **(I, K)** clathrin knocked down cells.

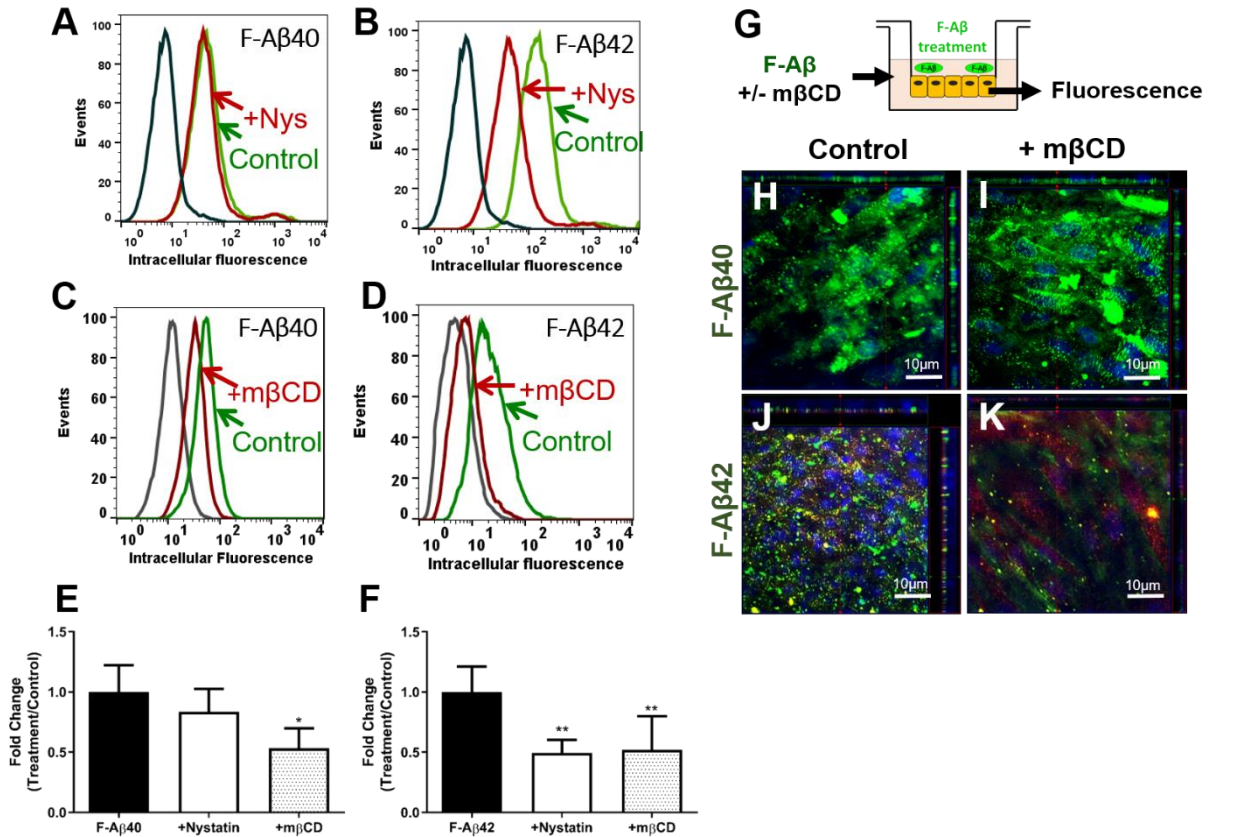


Figure 5. Role of lipid rafts on the internalization of F-Aβ peptides in hCMEC/D3 and

bovine brain microvascular endothelial (BBME) cells. (A-D) Histograms of hCMEC/D3 cells incubated with **(A)** F-Aβ40 and **(B)** F-Aβ42 in control and Nystatin (a specific inhibitor of lipid raft-caveolae endocytosis pathway) pretreated group, **(C)** F-Aβ40 and **(D)** F-Aβ42 in control and mβCD (a non-specific cholesterol chelator) pretreated group. **(E-F)** Fold change of median cellular fluorescence intensity from three replicate samples (n=3). *p<0.05, **p<0.01 compared to F-Aβ alone group, one-way ANOVA with Bonferroni post-tests **(G)** Experiment design of confocal microscopy study. Polarized BBME cell monolayers cultured on Transwell® filters were preincubated with or without methyl beta cyclodextrin (mβCD) in DMEM and then incubated with 1 μM F-Aβ peptides and/or 20 μg/ml AF633-Trf. **(H-K)** The z-series confocal micrographs demonstrating F-Aβ (green) fluorescence in **(H, J)** control cells and **(I, K)** mβCD treated cells. Images were shown in x-y (large square), x-z (top horizontal panel) and y-z (right vertical panel) plane.

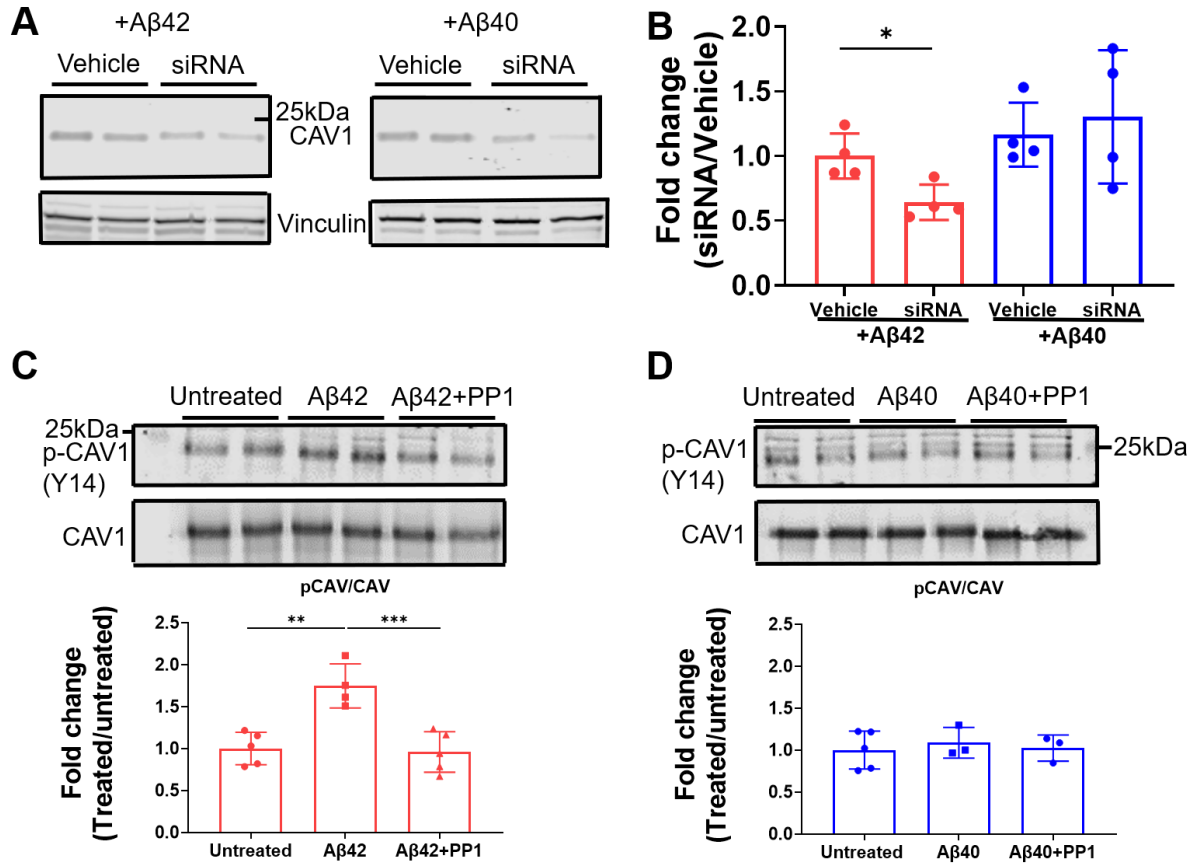


Figure 6. Tyrosine phosphorylation of caveolin-1 is induced by A β 42 but not by A β 40 and mediates A β 42 uptake. (A) Representative immunoblots confirming caveolin-1 knockdown using siRNA in hCMEC/D3 cells. (B) Intracellular A β accumulation in hCMEC/D3 cells transfected with caveolin-1 siRNA. * p <0.05, student's t-test. (C-D) Representative immunoblots and quantification of phosphorylated caveolin-1 (p-CAV1 (Y14)) and total caveolin-1 (CAV1) with (C) A β 42 and (D) A β 40 stimulation. ** p <0.01, *** p <0.005, one-way ANOVA with Bonferroni post-tests.

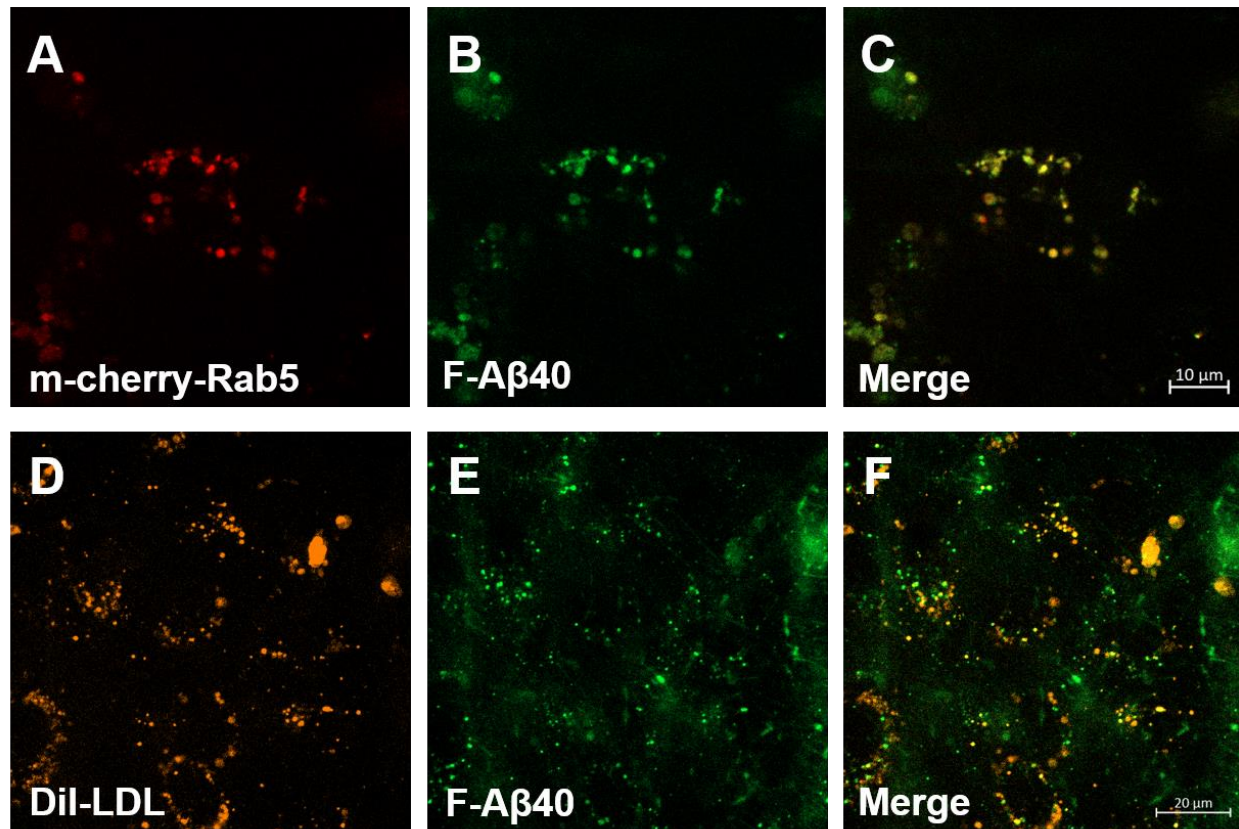


Figure 7. Accumulation of F-Aβ40 peptides in early and secondary endosomes in hCMEC/D3 cells. (A-C) Colocalization of F-Aβ40 with m-cherry fluorescent Rab5. hCMEC/D3 cells were transfected with m-cherry fluorescent Rab5 and then incubated with F-Aβ40 for 15 minutes. Then Live cell imaging was conducted after washing the cells with DMEM. **(A)** m-cherry fluorescent Rab5, **(B)** F-Aβ40, **(C)** Merge image of A and B. **(D-F)** Colocalization of F-Aβ40 with DiI-LDL. hCMEC/D3 cells were treated with 1 μM F-Aβ40 and DiI-LDL (15 μg/ml) for 60 minutes. Then Live cell imaging was conducted after washing the cells with DMEM. **(D)** DiI-LDL, **(E)** F-Aβ40, **(F)** Merge image of D and E.

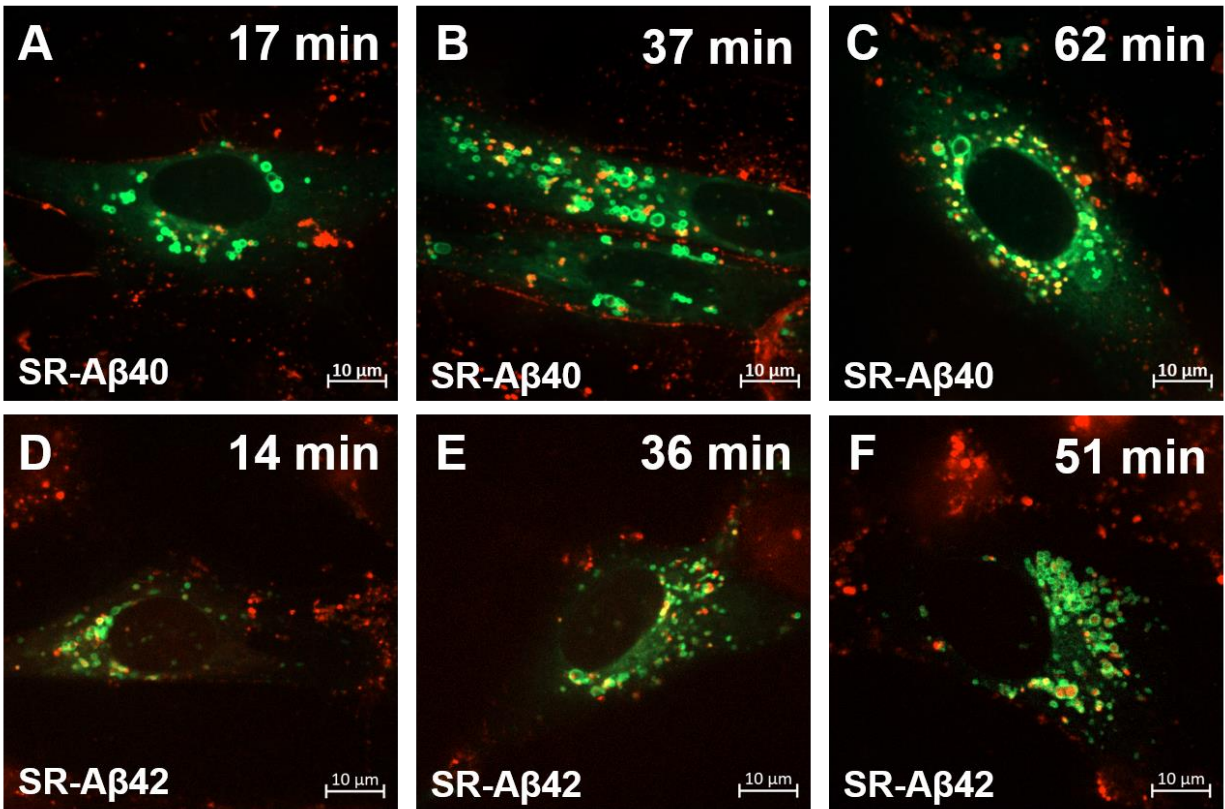


Figure 8. Time dependent accumulation of sulforhodamine labeled A β (SR-A β) peptides in late endosomes. hCMEC/D3 cells expressing GFP-Rab7 incubated with SR-A β for 15 minutes. Then the cells were washed with DMEM and imaged by live cell imaging up to 60 minutes. **(A-C)** Accumulation of SR-A β 40 following incubations at **(A)** 17min **(B)** 37 min **(C)** 62 min; **(D-E)** Accumulation of SR-A β 42 following incubations at **(D)** 14min **(E)** 36 min **(F)** 51 min.

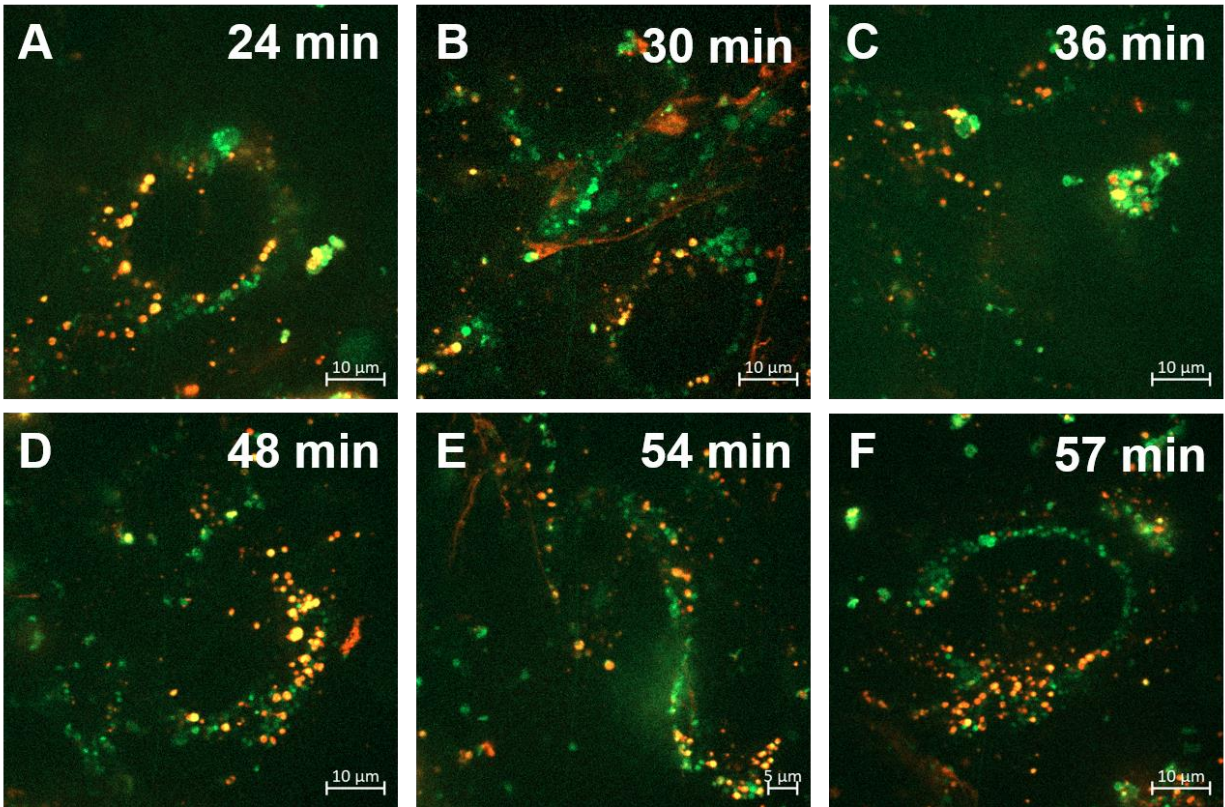
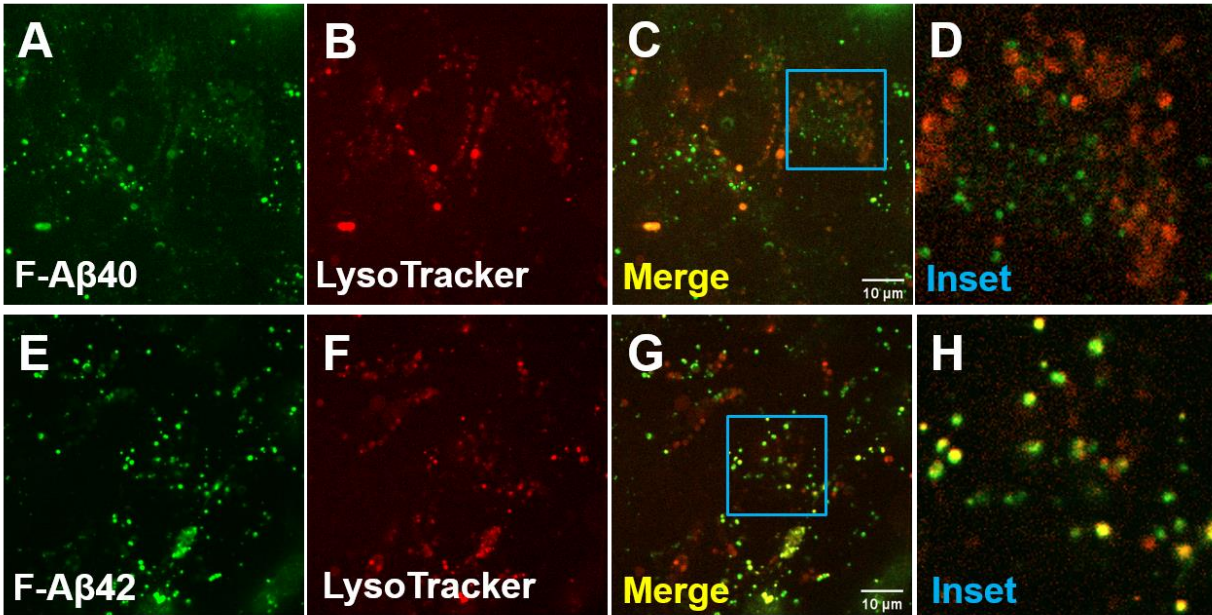


Figure 9. Time dependent accumulation of sulforhodamine labeled A β 40 peptides in recycling endosomes. hCMEC/D3 cells expressing GFP-Rab11 incubated with SR-A β 40 for 15 minutes. Then the cells were washed with DMEM and imaged by live cell imaging up to 60 minutes. **(A-C)** Accumulation of SR-A β 40 following incubations at **(A)** 24min **(B)** 30 min **(C)** 36 min **(D)** 48min **(E)** 54 min **(F)** 57 min.



I. Pearson correlation coefficient of Aβ and lysosome colocalization

Aβ species	Aβ40	Aβ42	Significance
PCC value	0.46±0.04	0.58±0.12	*

Figure 10. Accumulation of F-Aβ40 and F-Aβ42 peptides in lysosomes in hCMEC/D3 cells. Representative images showing colocalization of F-Aβ (green) and LysoTracker™ (red) in hCMEC/D3 cells. **(A-C)** F-Aβ40; **(E-G)** F-Aβ42; **(D, H)** Inset showing the magnified images of C & G. (I) Pearson correlation coefficient of F-Aβ and LysoTracker™. *p<0.05, student's t-test

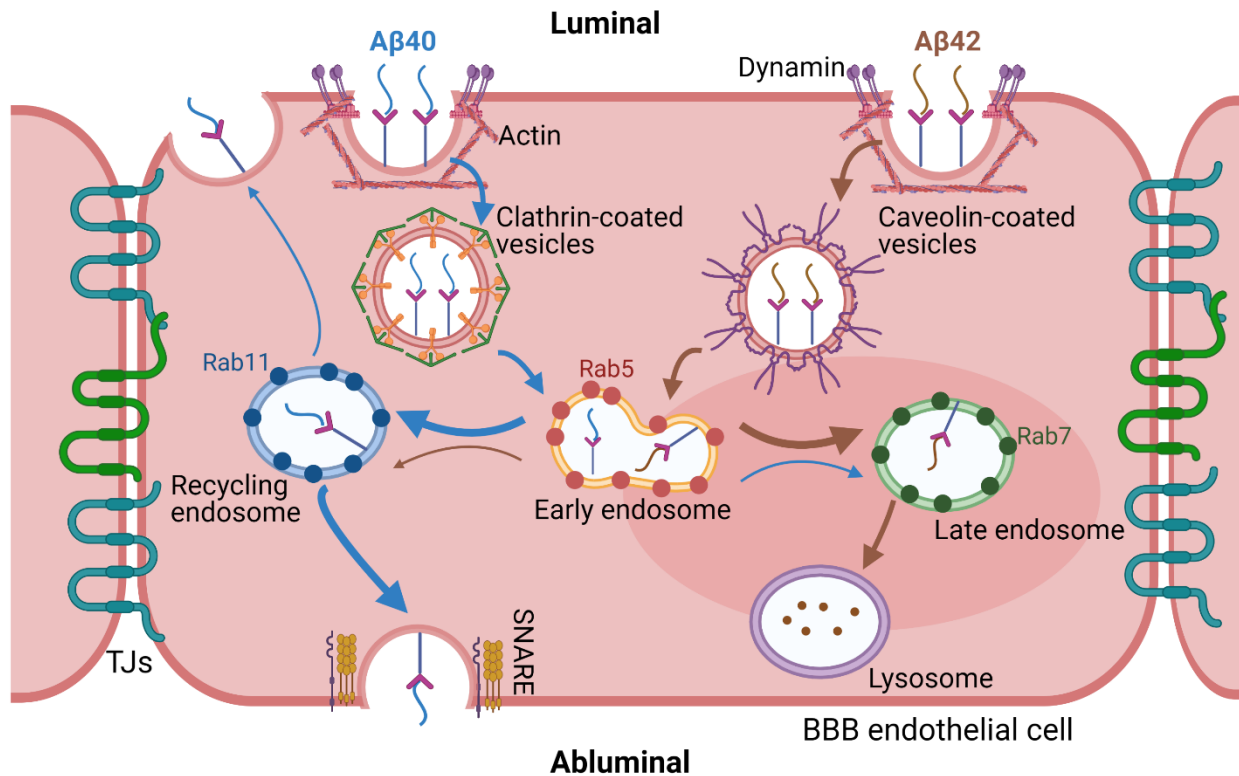


Figure 11. Summary of Aβ40 and Aβ42 endocytosis and intracellular trafficking pathway in the BBB endothelial cells. Both peptides were internalized via energy, dynamin and actin-dependent endocytosis. Endocytosis of Aβ40 was found to be clathrin-mediated whereas Aβ42 was majorly dependent on caveolae. Following endocytosis, the peptides were sorted by the endo-lysosomal system of endothelial cells. Aβ42 was found to accumulate more in the late endosomes and lysosomes (brown thick arrow) but Aβ40 was more likely to get transcytosed to the abluminal side (blue thick arrow) via recycling endosome and soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors (SNARE) protein facilitated exocytosis.

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