

## Endocytosis and intracellular processing of platelet microparticles by brain endothelial cells

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### Abstract

Platelet-derived microparticles (PMP) bind and modify the phenotype of many cell types including endothelial cells. Recently, we showed that PMP were internalized by human brain endothelial cells (HBEC). Here we intend to better characterize the internalization mechanisms of PMP and their intracellular fate. Confocal microscopy analysis of PKH67-labelled PMP distribution in HBEC showed PMP in early endosome antigen 1 positive endosomes and in LysoTracker-labelled lysosomes, confirming a role for endocytosis in PMP internalization. No fusion of calcein-loaded PMP with HBEC membranes was observed. Quantification of PMP endocytosis using flow cytometry revealed that it was partially inhibited by trypsin digestion of PMP surface proteins and by extracellular  $\text{Ca}^{2+}$  chelation by EDTA, suggesting a partial role for receptor-mediated endocytosis in PMP uptake. This endocytosis was independent of endothelial receptors such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and was not increased by tumour necrosis factor stimulation of HBEC. Platelet-derived microparticle internalization was dramatically increased in the presence of decomplexed serum, suggesting a role for PMP opsonin-dependent phagocytosis. Platelet-derived microparticle uptake was greatly diminished by treatment of HBEC with cytochalasin D, an inhibitor of microfilament formation required for both phagocytosis and macropinocytosis, with methyl- $\beta$ -cyclodextrin that depletes membrane cholesterol needed for macropinocytosis and with amiloride that inhibits the  $\text{Na}^+/\text{H}^+$  exchanger involved in macropinocytosis. In conclusion, PMP are taken up by active endocytosis in HBEC, involving mechanisms consistent with both phagocytosis and macropinocytosis. These findings identify new processes by which PMP could modify endothelial cell phenotype and functions.

**Keywords:** endothelial cells • inflammation • internalization • macropinocytosis • microparticles • phagocytosis

### Introduction

Microparticles (MP) are submicron membrane fragments released from many cell types upon activation or apoptosis. Their composition and functional properties vary with their cellular origin and the type of stimulus involved in their formation. Circulating MP mainly derive from platelets. Elevated levels of PMP have been

associated with numerous pathological states such as atherothrombosis [1] or infectious diseases [2, 3]. Platelet-derived microparticle interactions with various cell types and their functional consequences have been described in many studies. Indeed, PMP modulate biological functions of endothelial cells, leucocytes or haematopoietic cells *via* antigens, bioactive lipids or chemokines that they transport [4–11].

Less attention has been paid to the fate of PMP and neither their life span nor the mechanisms of their clearance have been clearly determined yet. Several groups suggested that PMP were cleared from the circulation very quickly following infusion into animal [12, 13] and, more recently, lactadherin has been identified as one of the mediators of the clearance of phosphatidylserine (PS)-expressing PMP by splenic macrophages [14].

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In a recent study, we showed that PMP were internalized by HBEC by an active process and induced changes in the surface phenotype of these cells that could have a pivotal role in the pathogenesis of cerebral malaria, an acute encephalopathy secondary to the infection by *Plasmodium falciparum* [15]. However, this internalization process had not been characterized. Here, using fluorescent markers for several endocytic compartments and various specific endocytosis inhibitors, we further characterize the intracellular sorting of PMP in HBEC and the mechanisms involved in this endocytosis. These analyses may help not only to elucidate the mechanisms by which PMP are taken up and degraded or recycled by cells, but also provide new insights in the regulation of the transport of biomolecules by PMP into target cells.

## Materials and methods

### Microvascular endothelial cells

Human brain endothelial cells, HBEC-5i, were derived by Dorovini-Zis *et al.* from small fragments of human cerebral cortex obtained from patients who had died of various causes [16]. These immortalized cells were then characterized and maintained in our laboratory, as described elsewhere [15, 17].

### Platelet microparticles

Platelet-derived microparticles were purified from *in vitro*-activated platelet supernatants and labelled with red PKH26 or green PKH67 (Sigma-Aldrich, St. Louis, MO, USA) as previously described [15]. Briefly, washed platelets from human blood were incubated for 45 min. at 37°C in the presence of calcium ionophore A23187 (10 µmol/l). Platelets and cell debris were removed by serial centrifugations (6 min. at 2000 × *g* and 2 min. at 13,000 × *g*), PMP were pelleted by centrifugation 60 min. at 20,800 × *g* and resuspended in PBS. Platelet-derived microparticles were then mixed volume to volume with PKH by gentle pipetting for 1 min. and washed with DMEM without phenol red (Gibco-Invitrogen-Life Technologies, Villebon sur Yvette, France). Alternatively, PMP were directly incubated with 10 µg/ml calcein AM (Invitrogen, Marseille, France) for 60 min. at 37°C and washed twice in PBS. Labelled PMP were counted by flow cytometry. Supernatants resulting from the final wash were used as a control.

In some experiments, to remove protease-sensitive glycoproteins from the surface of PMP, we performed digestion by 0.25% (w/v) trypsin (Gibco) for 30 min. at 37°C as described by Baj-Krzyworzeka *et al.* [18].

### Identification of the endocytic compartments containing PMP

Human brain endothelial cells grown to confluence on gelatin-coated 8-well labtek slides (Nunc) were incubated for 90 min. total incubation time at 37°C with PKH67-labelled PMP and washed to remove unbound PMP. LysoTracker Red DND-99 (50 nM; Invitrogen) or ER-Tracker Red (100 nM; Invitrogen), for the labelling of lysosomes or endoplasmic reticulum respectively, were added 30 min. before washing and fixation. For early endosome labelling,

HBEC were fixed with 4% (v/v) paraformaldehyde for 10 min. and permeabilized with Triton X-100 0.1% (v/v) in PBS 5 min. at room temperature. Non-specific binding was blocked by incubation for 30 min. in PBS containing 1% (w/v) BSA. Early endosome antigen 1 (EEA-1) was detected using a mouse monoclonal IgG1 to EEA-1 (clone 14, 1:1000; BD Transduction Laboratories, San Jose, CA, USA) revealed with Alexa-Fluor 555 conjugated goat anti-mouse secondary antibody (1:400; Invitrogen). Both primary and secondary antibodies were diluted in 1% (w/v) BSA in PBS and incubated for 1 hr at room temperature. Finally, cells were washed and kept in PBS for analysis. Samples were analysed by confocal laser scanning microscopy using an Axiovert 200 inverted microscope, equipped with a Zeiss LSM 510 scanning module and Zeiss LSM510 Software (Zeiss, Oberkochen, Germany).

### PMP endocytosis by HBEC and inhibition assays

To quantify PMP endocytosis by HBEC, cells were grown to confluence in 96-well plates and were either left unstimulated or incubated overnight with 10 ng/ml tumour necrosis factor (TNF). Cell monolayers were then washed in serum-free medium and further incubated for 90 min. at 37°C with PKH67-labelled PMP (2000/µl) in serum-free DMEM.

To evaluate the effect of serum in PMP endocytosis, medium was supplemented with 10% foetal calf serum (FCS; Gibco) decomplexed by heating for 30 min. at 56°C.

In some experiments, the calcium-chelator EDTA was added to the medium to a final concentration of 2.5 mM during endocytosis assay to investigate the role of extracellular calcium in PMP endocytosis.

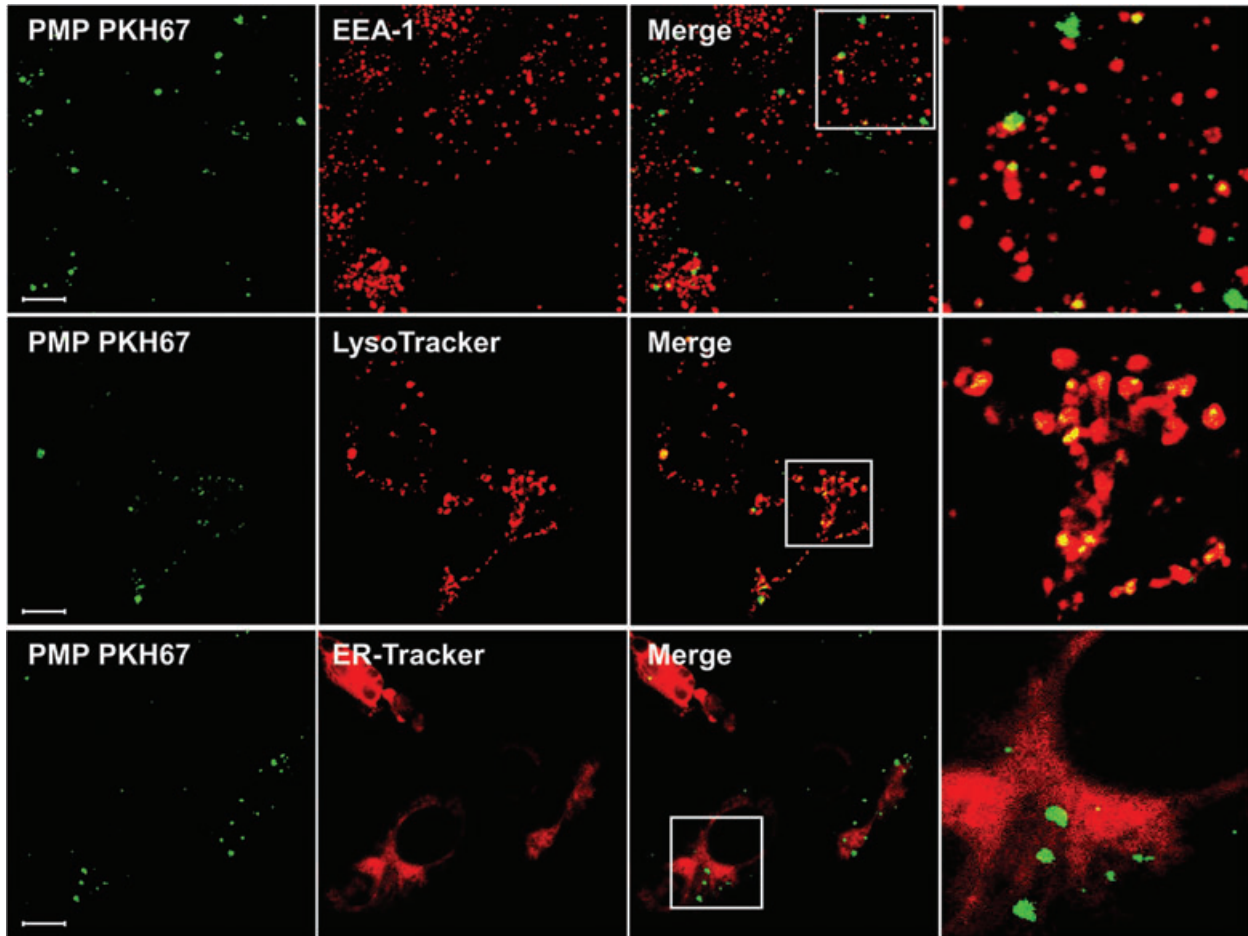
To evaluate the role of endothelial surface adhesion receptors in PMP internalization, HBEC were first pre-incubated for 30 min. at 4°C with mAbs against vascular cell adhesion molecule-1 (VCAM-1, CD106, clone 1G11, IgG1) and intercellular adhesion molecule-1 (ICAM-1, CD54, clone 84H10, IgG1) purchased from Beckman Coulter Immunotech. An irrelevant isotype-matched mAb (clone 679.1Mc7) was used as control.

To evaluate the effects of specific endocytosis inhibitors, HBEC were first pre-incubated with the following inhibitors in serum-free medium for 30 min. at 37°C: 10 µM cytochalasin D, 10 mM methyl-β-cyclodextrin and 250 µM amiloride. Cytochalasin D and amiloride were kept in the medium during the endocytosis assay. To avoid any effect of cholesterol depletion on PMP integrity, a washing step to remove methyl-β-cyclodextrin from the prior co-incubation medium was necessary before the addition of PMP and the endocytosis assay was carried out in the absence of this inhibitor. Cells were incubated with 0.5 mg/ml 70 kD-dextran (Sigma-Aldrich) at 37°C for 30 min. as a positive control for macropinocytosis.

Each inhibitor and EDTA were tested at different concentrations to establish working concentrations for maximum inhibition without toxic effects on cells. After incubation, HBEC were washed, detached by a short trypsin treatment and analysed for fluorescence by flow cytometry. To assess effective activation of HBEC by TNF, detached HBEC were analysed for surface up-regulation of VCAM-1 and ICAM-1 by flow cytometry as previously described [17]. For all these experimental conditions, flow cytometry analyses were performed with live cells using an Epics XL flow cytometer (Beckman Coulter Immunotech). Data were obtained and analysed using System2 software (Beckman Coulter Immunotech). Live cells were gated by forward/side scattering from a total of 10,000 events.

### Statistical analyses

Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test and are presented as continuous variables as



**Fig. 1** PMP are present in early endosomes and lysosomes. HBEC were incubated with PKH67-labelled PMP (green) or with PMP supernatants as a negative control for 90 min. at 37°C. HBEC were labelled with mouse anti-EEA-1 IgG revealed with Alexa 555-coupled goat antimouse IgG (top, red), with LysoTracker (middle, red) or with ER-Tracker (bottom, red). Cells were then analysed by confocal microscopy. For each selected sample, 15 optical sections separated by 0.6 nm steps were analysed. The lens used was a Zeiss Plan-Apochromat 63×/1.40 oil immersion objective lens. Inserts of the merged images are represented in the right column. Bars: 10 μm.

mean ± S.D. Means between two groups were compared using a two-tailed unpaired Student's *t*-test. *P* values less than 0.05 were accepted as significant. All statistical analyses were performed with GraphPad Prism version 4.0 for Windows Software.

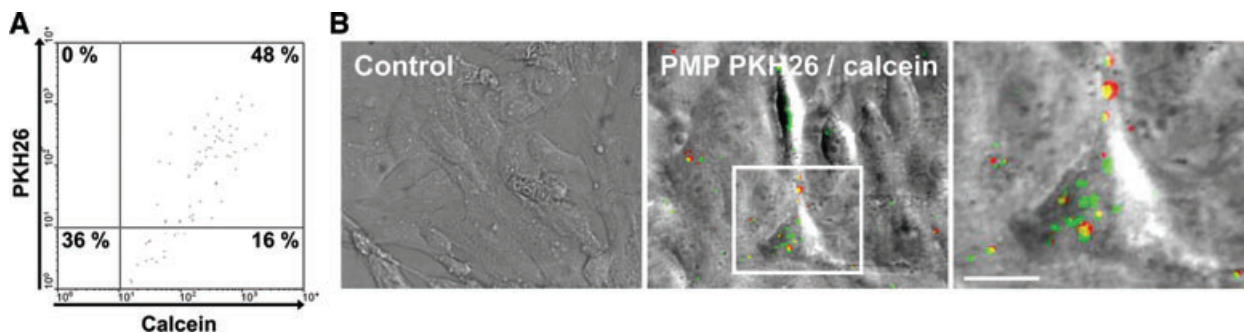
## Results

### Subcellular localization of internalized PMP

We previously demonstrated that PMP were internalized by HBEC in a time-dependent and temperature-sensitive process [15]. Once internalized, PMP were found to be surrounded by membranes

labelled with Alexa-555 coupled wheat germ agglutinin, suggesting that PMP moved into large intracellular vesicles but did not stay along the plasma membrane.

To evaluate the potential role of endocytosis in PMP uptake, we first identified the intracellular vesicles in which PMP were processed after internalization. Using confocal microscopy, the cell distribution of PKH67-labelled PMP was compared to that of endosomal structures, as revealed by EEA1 and LysoTracker red staining. As shown in Figure 1, after 90 min. of co-incubation, 15% of PMP were found in EEA1-positive vesicles (top) and most PMP co-localized with LysoTracker red fluorescence in HBEC (middle). These data clearly indicate that PMP are internalized by an endocytic process and that, after uptake, PMP traffic along the endosomal compartment through early endosomes towards more acidic lysosomes. Endocytosed PMP showed no colocalization



**Fig. 2** Co-localization of PMP cytoplasmic content and PMP-derived membranes inside the target cell. PMP were double-labelled with membrane dye PKH26 (red) and calcein AM that only becomes green fluorescent once hydrolysed by cytoplasmic esterases. **(A)** Analysis of PMP double-labelling by flow cytometry. Number in the upper right quadrant represents the percentage of double-labelled PMP. **(B)** PMP were then incubated with HBEC for 90 min. at 37°C. PMP supernatant was used as a negative control. Cells were washed, fixed with PFA 2% (v/v) and analysed by confocal microscopy. Merged images showing transmitted light and green and red channels are represented. Insert of the merged image is represented in the right column. Bar: 10  $\mu$ m.

with ER-Tracker staining (bottom), indicating that endocytosed PMP did not enter the retrograde-transport pathway to the endoplasmic reticulum at that time.

### Detection of PMP fusion with HBEC membrane by calcein release

To search for PMP fusion with the endothelial membrane and to detect a possible PMP fragmentation once internalized, PMP content was stained with soluble calcein AM (green) in addition to the membranous PKH26-labelling (red). After staining, 48% of PMP were double labelled for both calcein and PKH26 (Fig. 2A). Figure 2B shows that, after PMP internalization by HBEC, no PKH26 was transferred from PMP to endothelial membrane and no entrapped calcein was released within the cytosol, suggesting that PMP content did not diffuse inside the target cell but remained co-localized with PMP membranes. This observation clearly indicates that PMP internalization by HBEC does not involve the fusion of PMP lipids with the endothelial plasma membrane but, rather, an uptake of PMP material with subsequent localization in intracellular vesicles.

### Mechanisms of PMP endocytosis

To further characterize PMP internalization by HBEC, we tested the effect of different modulators added just before or during the internalization assay and quantified the uptake of PKH67-labelled PMP by flow cytometry (Fig. 3A). Firstly, PMP internalization was only reduced by 38% after trypsin digestion (Fig. 3B), indicating that, in addition to the trypsin-sensitive protein components of PMP, other trypsin-resistant components such as lipids can be responsible for their interaction with HBEC. Secondly, pre-treatment of HBEC with EDTA resulted in a significant 33% decrease of PMP uptake (Fig. 3B), suggesting that this phenomenon is partially cal-

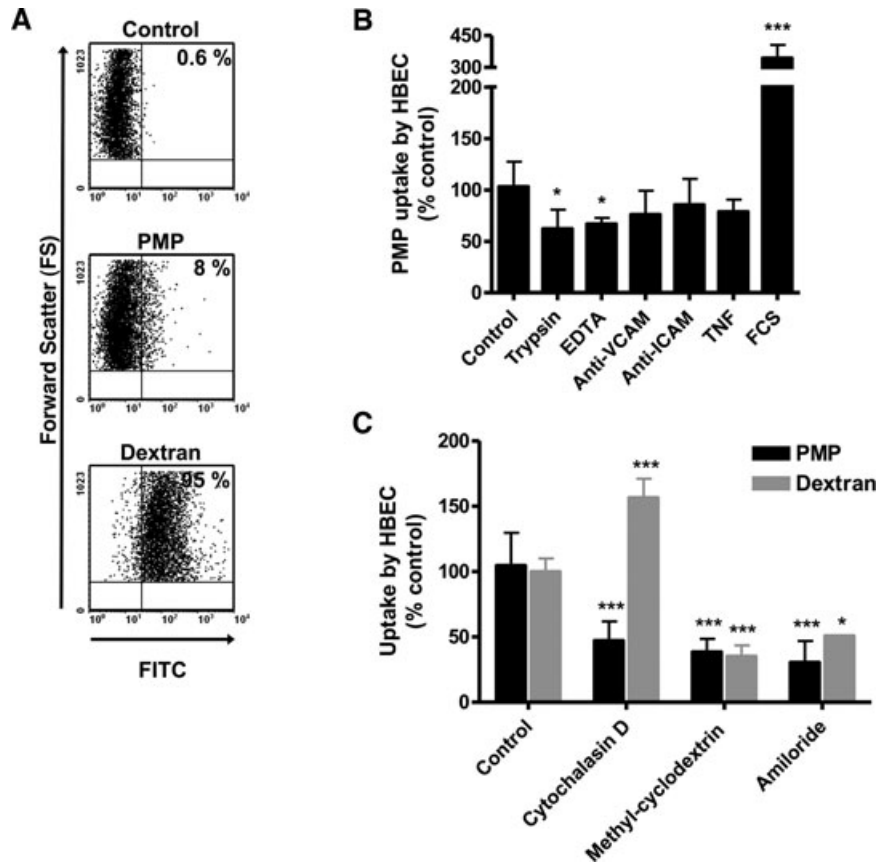
cium dependent. Thirdly, we showed that PMP internalization was not affected when HBEC were first incubated with mAbs against VCAM-1 or ICAM-1. Moreover, PMP internalization was not increased in TNF-pre-stimulated HBEC as compared to resting cells (Fig. 3B). This suggests that PMP internalization is not mediated by TNF-up-regulated adhesion receptors such as VCAM-1 or ICAM-1. Surprisingly, in the presence of decompartmented FCS, there was a threefold increase in PMP uptake (Fig. 3B), suggesting a role for PMP opsonization by decompartmented FCS in their entry into HBEC.

To determine which of the several possible cellular uptake mechanisms were responsible for PMP internalization, the effects of three different endocytosis inhibitors were examined. These inhibitors were also tested on the entry of 70-kD FITC-dextran, a marker of macropinocytosis, for comparison. Cytochalasin D was used to inhibit actin elongation and the formation of microfilaments and microtubules, methyl- $\beta$ -cyclodextrin to deplete membrane cholesterol and inhibit lipid raft-dependent pathways, and amiloride was tested to inhibit the  $\text{Na}^+/\text{H}^+$  exchanger required for macropinocytosis [19]. In HBEC, 70 kD FITC-dextran endocytosis was reduced by methyl- $\beta$ -cyclodextrin and amiloride, as expected for macropinocytosis (Fig. 3C, grey bars). Cytochalasin D was found to produce an unexpected increase in dextran entry into HBEC, indicating an alteration in macropinocytosis with this inhibitor. In contrast, all drugs tested were found to partially inhibit the entry of PMP into HBEC (Fig. 3C, black bars).

## Discussion

A growing number of studies report the cellular uptake of MP by mononuclear haematopoietic cells, monocytes, neutrophils, plasmacytoid dendritic cells [18, 20–23] and by endothelial cells [15, 24–26]. However, the mechanisms involved in this process have not been clearly defined yet.

**Fig. 3** PMP endocytosis assay. HBEC were incubated with PKH67-labelled PMP or 70 kD FITC-dextran for 90 min. at 37°C. PMP supernatant was the negative control. Fluorescent cells were quantified by flow cytometry. **(A)** Numbers in the upper right quadrants represent the percentage of fluorescent cells. **(B)** HBEC were either co-incubated with trypsin-treated PMP, co-incubated with PMP in the presence of EDTA, pre-incubated with blocking mAbs against ICAM-1 or VCAM-1, pre-stimulated with TNF before PMP co-incubation or co-incubated with PMP in the presence of decompartmented FCS. **(C)** Effects of specific endocytosis inhibitors on PMP or 70 kD FITC-dextran uptake. HBEC were pre-treated with cytochalasin D, methyl- $\beta$ -cyclodextrin or amiloride for 30 min. prior to adding PKH67-labelled PMP for 90 min. or 70 kD FITC-dextran for 30 min. **(B, C)** Results are presented as the percentage of PMP uptake (mean fluorescence intensity) compared to the 37°C-positive control (mean  $\pm$  S.D.). A two-tailed, unpaired Student's *t*-test was used for comparison of means; \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001.



In this study, we have examined the intracellular localization of PMP internalized by brain microvascular endothelial cells and attempted to decipher the pathways involved in this phenomenon. Using markers for endosomal compartments, we demonstrated for the first time that internalized PMP were distributed within endosomes and lysosomes after cell entry, supporting the fact that endocytosis was the predominant route of PMP entry into endothelial cells. This major finding differs from the recent work by Terrisse *et al.* who did not find any co-localization of endocytosed endothelial-derived MP with markers of early or late endosomes after 2 hrs of co-incubation with HUVEC [25], suggesting that either kinetics or pathways for MP intracellular processing might vary between different endothelial cell types.

Although PMP were efficiently endocytosed by endothelial cells, they did not fuse with their plasma membrane because no diffusion of calcein-labelled PMP content occurred inside the target cell cytosol. This is consistent with the observation of Aharon *et al.* who found that monocyte-derived MP bound to and were ingested by HUVEC without any fusion of MP with the cell membrane [24] but contrasts with the fusion of monocyte-derived MP with activated platelets described by del Conde *et al.* [27]. These results suggest that MP interaction might involve different mechanisms depending on the target cell type.

Endocytosis may occur through several distinct mechanisms, usually divided into two broad categories: phagocytosis, a process restricted to specialized mammalian cells, and pinocytosis, which occurs in all mammalian cells and encompasses macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis [28]. Although clathrin- and caveolin-dependent endocytosis involve the uptake of particles smaller than 100 nm in diameter, phagocytosis and macropinocytosis involve the uptake of larger volumes. Because MP are defined as particles with a diameter varying between 100 and 1000 nm [6], we focused on the potential role for phagocytosis or macropinocytosis in PMP uptake.

Although phagocytosis requires cell-surface recognition of particles and proceeds *via* sequential engagement of receptors, macropinocytosis occurs *via* non-selective membrane uptake. We thus tried to determine which type of MP surface components and endothelial receptors could be involved in MP endocytosis by HBEC. The partial inhibition of PMP internalization after treatment of PMP with trypsin indicates that protease-sensitive components of PMP, such as surface glycoproteins, play a minor role in PMP binding and further internalization but rather supports a role for other components such as lipids. PMP uptake was slightly inhibited by EDTA, suggesting that  $Ca^{2+}$ -dependent (usually

receptor-mediated) endocytosis was partly involved in PMP uptake. Because platelet adhesion and fusion to TNF-activated brain endothelial cells depends on ICAM-1 up-regulation on endothelial membranes and LFA-1 expression on platelets [29–31], we wanted to evaluate the role of ICAM-1 in PMP binding and internalization by HBEC. Pre-incubation of HBEC with mAb against ICAM-1 or VCAM-1 failed to inhibit PMP uptake. Furthermore, TNF activation of HBEC, which increases both ICAM-1 or VCAM-1 expression on HBEC surface [17], did not increase PMP internalization, implying that these adhesion receptors are not required for PMP endocytosis. On the other hand, the increased uptake of PMP in the presence of decomplemented FCS suggests that PMP can be opsonized by FCS components other than proteins from the complement cascade supporting a role for phagocytosis.

The composition of MP is related to their cellular origin and to the type of stimulus involved in their formation; therefore, the cellular events required for their internalization may differ between both target cells and MP cellular origins. Nonetheless, one common feature of MP is the exposure on their external leaflet of PS, also present on the external surface of apoptotic cells and apoptotic platelets. PS exposure [32, 33] and recognition by many receptors such as scavenger receptors present on macrophages [34] are crucial for phagocytosis and clearance of apoptotic platelets and are probably also relevant in the clearance of PMP. Indeed, three recent studies showed that MP endocytosis by either HUVEC [25, 26] or macrophages [35] was significantly inhibited in the presence of annexin V suggesting a major role for PS. Flaumenhaft proposed that PS exposure on PMP may support opsonization by C3b. C3b-bearing PMP could subsequently bind to erythrocytes through complement receptor CR1 and be delivered to scavenger endothelium in the liver and spleen by receptor-mediated endocytosis [13]. Scavenger receptors have been evoked to be involved in the removal from the circulation of red blood cell-derived MP by Kupffer cells in the liver [36]. Both scavenger receptors [37, 38] and PS receptor (PSR) [39] are expressed on microvascular endothelial cells but PSR is highly unlikely to be involved in PMP endocytosis under basal conditions because PSR does not appear to be constitutively expressed on the cell surface [39]. Other opsonins such as  $\beta_2$ -glycoprotein I [35], IgM [40] or lactadherin [14] have been involved in MP phagocytosis by macrophages. Recently, Terrisse *et al.* showed that HUVEC internalized endothelium-derived MP through a process involving anionic phospholipids, lactadherin and  $\alpha_v\beta_3$  integrin but not CD36 [25]. The importance of this potential interaction in our experimental model warrants further investigations especially as HBEC-5i express constitutive  $\alpha_v\beta_3$  integrin but no CD36 on their surface [17].

Both phagocytosis and micropinocytosis are characterized by extensive membrane reorganization and are reduced by cytochalasin D treatment [28]. Thus, the reduction of PMP entry into

cells by cytochalasin D does not allow us to identify one of the two processes as a major pathway for PMP entry into HBEC. The decrease of PMP uptake by the cholesterol depleting agent methyl- $\beta$ -cyclodextrin shows that PMP endocytosis depends on cholesterol content and suggests a role for macropinocytosis because cholesterol is required for membrane ruffling and macropinocytosis [41]. It should be stressed out that, in addition to cholesterol depletion, methyl- $\beta$ -cyclodextrin has pleiotropic effects on different membrane components, such as phospholipids, that could have a potential role in PMP endocytosis. The incomplete inhibition of PMP entry by the  $\text{Na}^+/\text{K}^+$  transporter inhibitor amiloride indicates that endocytic uptake of PMP occurs partially by non-selective macropinocytosis [19]. The unique susceptibility of macropinocytosis to inhibitors of  $\text{Na}^+/\text{H}^+$  exchange has been extensively used to differentiate it from other types of endocytosis. However, caution should be made in interpreting these data because the inhibition of macropinocytosis by amiloride could also result from its effects on cell pH [42].

Phagocytosis of particles, apoptotic bodies and aged erythrocytes is predominately attributed to macrophages, neutrophils and dendritic cells. However, several studies have described the uptake of large objects by endothelial cells, including apoptotic bodies [43–46] and MP [24–26]. More surprising is the endocytic activity of endothelial cells derived from the brain. Indeed, the most important function of endothelial cells that line the brain capillaries is to form the blood–brain barrier (BBB), which physically separates blood components from the brain and the cerebrospinal fluid. This barrier is based, in part, on a paucity of vesicular activity within the brain microvascular endothelial cells that limits endocytosis. However, endocytosis can be modulated by vasoactive substances, such as angiotensin and bradykinin [47], and cytokines, such as TNF [48], and may dramatically increase in disease states, rendering the BBB less restrictive for blood-borne proteins and cells.

Understanding PMP endocytotic process could provide essential clues that may reveal how endothelial cells can differentially regulate the transport of different components across the BBB. This is likely to shed light on the processes underlying numerous neurological disorders during which an increased production of PMP at the interface of the BBB occurs. For example, PMP carry agents implicated in neurodegenerative disorders such as amyloid  $\beta$ -protein precursor present in amyloid plaques in Alzheimer's disease [49] and PMP could actively transport these agents across the BBB [50].

Overall, our findings provide new mechanisms by which MP interact with endothelial cells and could modify their phenotype and functions. This could help understand the role of MP in the broad range of physiological and pathological processes during which MP levels are increased. MP uptake by endothelial cells could also participate in MP clearance from the circulation.

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## Authors' contributions

DF performed the research. DF, FEA, AM, GEG and VC designed the research, analysed the data and wrote the paper. MCA, GC and TF provided material and analytical tools.

## Conflict of interest

The authors confirm that there are no conflicts of interest.

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