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Flow shear stress differentially regulates endothelial uptake of nanocarriers targeted to distinct epitopes of PECAM-1

Jingyan Han [a,d], Vladimir V. Shuvaev [a], Peter F. Davies [b], David M. Eckmann [c], Silvia Muro [e], Vladimir R. Muzykantov [a,*]

[a] Department of Pharmacology and Center for Translational Targeted Therapeutics and Nanomedicine of the Institute for Translational Medicine and Therapeutics, University of Pennsylvania, Philadelphia, PA19104, USA
[b] Department of Pathology & Lab Medicine and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA19104, USA
[c] Department of Anesthesiology and Critical Care, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA19104, USA
[d] Vascular Biology Section, Department of Medicine, Boston University, Boston, MA 02421, USA
[e] Fischell Department of Bioengineering, University of Maryland, College Park, MD 20742, USA

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ABSTRACT

Targeting nanocarriers (NC) to endothelial cell adhesion molecules including Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1 or CD31) improves drug delivery and pharmacotherapy of inflammation, oxidative stress, thrombosis and ischemia in animal models. Recent studies unveiled that hydrodynamic conditions modulate endothelial endocytosis of NC targeted to PECAM-1, but the specificity and mechanism of effects of flow remain unknown. Here we studied the effect of flow on endocytosis by human endothelial cells of NC targeted by monoclonal antibodies Ab_{62} and Ab_{37} to distinct epitopes on the distal extracellular domain of PECAM. Flow in the range of 1–8 dyn/cm^2, typical for venous vasculature, stimulated the uptake of spherical Ab/NC (~180 nm diameter) carrying ~50 vs 200 Ab_{62} and Ab_{37} per NC, respectively. Effect of flow was inhibited by disruption of cholesterol-rich plasmalemma domains and deletion of PECAM-1 cytosolic tail. Flow stimulated endocytosis of Ab_{62}/NC and Ab_{37}/NC via eliciting distinct signaling pathways mediated by RhoA/ROCK and Src Family Kinases, respectively. Therefore, flow stimulates endothelial endocytosis of Ab/NC in a PECAM-1 epitope specific manner. Using ligands of binding to distinct epitopes on the same target molecule may enable fine-tuning of intracellular delivery based on the hemodynamic conditions in the vascular area of interest.

1. Introduction

Cellular uptake of targeted nanocarriers (NC) for drug delivery is regulated by parameters of carrier design (e.g., selection of target epitopes), as well as target cell phenotype and factors associated with the cellular microenvironment [1–4]. Thus, experimental models for delivery of NC should account for target cell conditions in vivo [5–8]. For example, the functional status of endothelial cells lining the vascular lumen, an important target for drug delivery, is greatly influenced by fluid shear stress of blood flow that varies under physiological and pathological conditions [9]. The role of blood rheology and hydrodynamics in NC binding to endothelium is extensively studied [10–15]. In contrast, relatively little is known about the role of these factors in the intracellular uptake of nanoparticles bound to specific endothelial surface molecules. Several lines of evidence suggest an important role of flow in the regulation of endocytosis of macromolecules and particles, such as albumin, non-targeted nanoparticles (e.g., quantum dots, SiO_2^- nanoparticles [16]), and nano- and micro-sized hydrogel spheres [17].

However, the role of hemodynamics in endocytosis of NC targeted to endothelial cells by affinity ligands including antibodies (i.e., Ab/NC) remains enigmatic. It is plausible that flow regulates this process in a ligand-specific fashion, since nature of the binding site and mode of ligand engagement control the mechanism of endocytosis. Recent studies in vitro and in vivo revealed that flow conditions modulate endothelial endocytosis of Ab/NC targeted to the cell adhesion molecules ICAM-1 and PECAM-1 [12,18]. Drug delivery using Ab/NC targeted to these determinants improves therapeutic effects of experimental drugs and biotherapeutics in animal models [19–22]. This justifies efforts directed towards extending our knowledge of the factors controlling intracellular delivery of NC targeted to these molecules. PECAM-1 antibodies bind to endothelial cells but do not accumulate significantly in the intracellular compartments [23,24]. In contrast, the multivalent binding of NCs coated with PECAM-1 antibody (e.g., Ab/NC) leads to intracellular uptake mediated by the pathway known as CAM-endocytosis, distinct from clathrin- or caveolae-mediated endocytosis, phagocytosis and macropinocytosis [23,25]. Furthermore, recent studies showed that shear stress stimulates endocytosis of PECAM-1-targeted Ab/NC [18].

* Corresponding author.
E-mail address: muzykantov@mail.med.upenn.edu (V.R. Muzykantov).

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However, previous studies revealed that under standard static cell culture conditions, human endothelial cells differentially internalize Ab/NC targeted to specific PECAM-1 epitopes: e.g., they internalize Ab/NC targeted by monoclonal antibody 62 (Ab_{62}) but not by monoclonal antibody 37 (Ab_{37}), which both are directed to distinct epitopes located in the distal Ig-like domain of PECAM-1 (i.e., Ab_{62}/NC and Ab_{37}/NC, respectively) [26]. These findings imply that control of endothelial internalization by physiological factors including flow may be different for Ab/NC targeted to distinct epitopes of PECAM-1. In the present study we have investigated whether this effect of flow is epitope-specific.

2. Results

2.1. Flow differently modulates endothelial internalization of Ab/NC targeted to distinct PECAM-1 epitopes

Binding to and uptake by target cells are proportional to Ab/NC's avidity, controlled by antibody affinity and number on a NC surface. Coupling ~200 antibody molecules per 100 nm particle provides nearly maximal surface density of monolayer IgG coating [27]. We started with assessing the uptake by endothelial cells of such NC coated by Ab_{37} vs Ab_{62}, either at static conditions or 30 min after exposure to non-pulsatile laminar flow generating a flow shear stress of 4 dyn/cm^2. Double-label fluorescent microscopy with secondary fluorescent antibody allows to distinguish cell surface-bound vs intracellular fluorescent nanocarriers (Fig. 1A). We found that flow almost doubles uptake of Ab_{37}/NC, which are barely internalized by static endothelial cells (Fig. 1A & B). However, flow rather trivially augmented uptake of Ab_{62}/NC, which are effectively internalized by static cells (Fig. 1A & B).

The binding of Ab_{37}/NC to endothelial cells was markedly lower than that of Ab_{62}/NC (Supplemental table 2 and Fig. 1A and C). Noteworthy, flow stimulated internalization of Ab_{37}/NC without changing its binding (Fig. 1B and C). The data of uptake of Ab/NC incubated at different concentrations with static cells further distinguished binding vs internalization. As expected, endothelial binding of both types of Ab/NC increased proportionally to their concentration (Fig. 2A). Importantly,

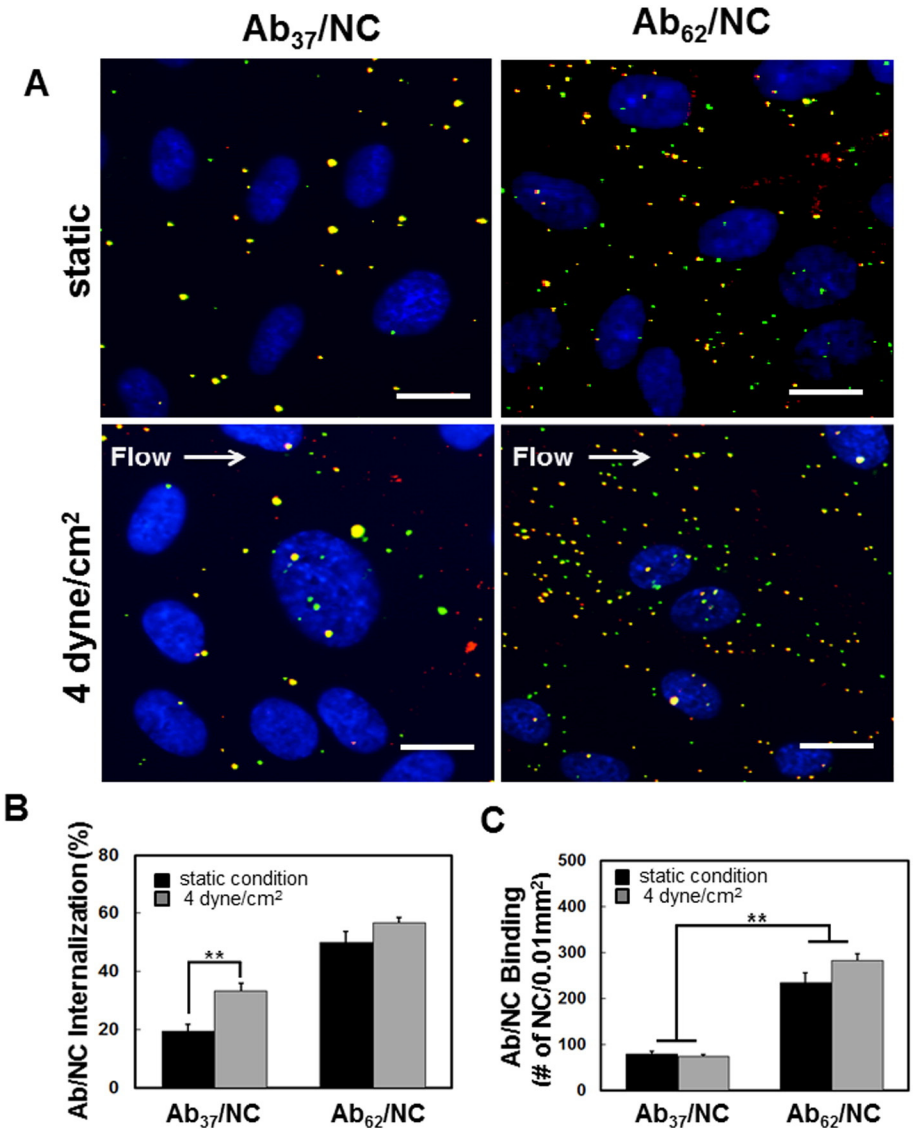

Fig. 1. Endothelial binding and internalization of nanocarriers coated by Ab_{37} and Ab_{62}. *A*, Representative fluorescence images showing endothelial binding (total particles) and internalization (green particles) of NC coated with Ab_{37} (left panel) versus Ab_{62} (right panel) at maximal Ab density on the surface of NC (200Abs/NC) under static and laminar flow conditions. Scale bar is 20 μm. *B* and *C*, confluent endothelial cells were incubated or exposed to flow (4 dyn/cm^2) with Ab/NC at the NC concentration of 2×10^9/ml for 30 min at 37 °C. Flow stimulates internalization of Ab_{37}/NC (*B*). Fewer Ab_{37}/NC were bound to endothelial cells under static and flow conditions (*C*). The percentage of Ab/NC internalized into endothelial cells (*B*) and total number of Ab/NC bound to endothelial cells (*C*) in each image field (0.01 mm^2) were quantified by fluorescence microscopy and presented as Mean ± S.E. (n = 8). $^*p < 0.05$, $^{**}p < 0.01$.

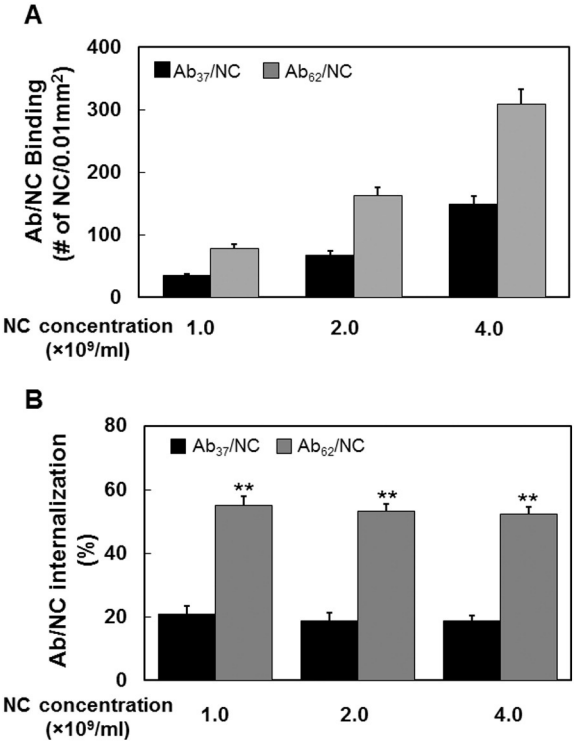

Fig. 2. Endocytosis of Ab_{37}/NC and Ab_{62}/NC is independent of the total number of Ab/NC bound to endothelial cells. *A*, Ab_{37}/NC and Ab_{62}NC (200 Ab/NC) at different concentrations (1, 2, and 4×10^9 particles/ml) were incubated with confluent endothelial cells for 30 min. The total number of Ab_{37}/NC (black bar) and Ab_{62}/NC (gray bar) bound to cells and their internalization (insert) in each image field (0.01 mm^2) were quantified by fluorescence microscopy.

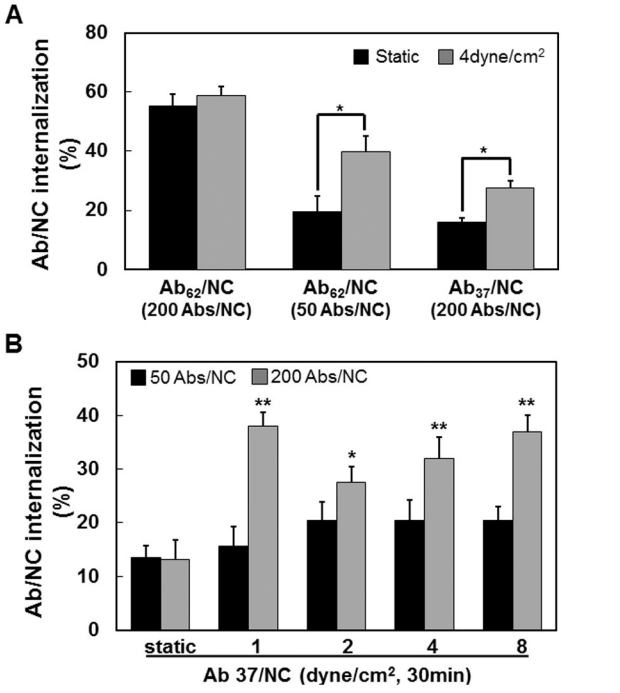

Fig. 3. Fluid shear stress stimulates endocytosis of Ab_{37}/NC and Ab_{62}/NC: role of Ab/NC avidity. *A*, internalization of Ab_{62}/NC (50 and 200 Abs/NC) and Ab_{37}/NC (200 Abs/NC) under static and flow conditions (incubation or perfusion at 4 dyn/cm^2 for 30 min) were quantified. *B*, internalization of Ab_{37}/NC at low (50 Abs/NC) and high (200 Abs/NC) antibody density over particle surface in endothelial cells was quantified under static or flow (1, 2, 4, 8 dyn/cm^2, 30 min) conditions $^*p < 0.05$, $^{**}p < 0.01$ in comparison with static groups.

binding of Ab_{37}/NC at high concentration exceeded that of Ab_{62}/NC at low concentration. However, the level of Ab_{37}/NC internalization remained consistently three-fold lower than that of Ab_{62}/NC, i.e., ~20% vs 60% (Fig. 2B). Taken together, these data indicate that both Ab/NC internalization and its modulation by flow do not necessarily correlate directly with number of cell-bound Ab/NC. This is important in the context of the effects of flow in endocytosis of Ab/NC (see Discussion section).

However, antibody surface density, which controls valence and avidity of binding, is an important factor in flow-mediated stimulation of the Ab/NC uptake. First, reduction of surface density of Ab_{62}/NC coating from ~200 to 50 antibody molecules per particle decreased endothelial endocytosis to the level comparable with that of Ab_{37}/NC carrying ~200 antibody molecules (Fig. 3A). Second, flow stimulated endocytosis of Ab_{62}/NC with low Ab density similarly to that of Ab_{37}/NC with high Ab density. Finally, flow ranging from 1 to 8 dyn/cm^2 showed a trend to stimulate proportionally endocytosis of Ab_{37}/NC carrying ~50 Ab molecules, but this trend did not reach statistical significance (Fig. 3B).

These data show that flow stimulates endocytosis of Ab/NC within a restricted range of Ab/NC avidity to endothelium. For Ab_{62}/NC and Ab_{37}/NC, it is close to that provided by low and high antibody density, respectively. Exceeding this empirical range, e.g., by using high antibody density Ab_{62}/NC results in a high internalization rate overshadowing the effect of flow, whereas falling below this range (e.g., by using low Ab density Ab_{37}/NC) inhibits the internalization beyond that which is salvageable by stimulatory flow effect.

2.2. Cholesterol-rich plasmalemma domain(s) and PECAM-1 cytosolic tail mediate stimulation of endocytosis of Ab_{62}/NC and Ab_{37}/NC by flow

Next, we addressed cellular mechanisms involved in stimulation of endocytosis by flow. Here we focused on two factors: sensing of flow by cholesterol-rich domains in the plasmalemma and signaling via PECAM-1 anchoring molecule.

First, we found that cholesterol sequestration by methyl-β-cyclodextrin, which affects the lipid rafts and caveolae, abrogated stimulatory effect of flow on endothelial endocytosis of Ab_{37}/NC and Ab_{62}/NC (high and low antibody density formulations, respectively), without affecting their internalization under static conditions (Fig. 4).

Second, we tested whether signaling via PECAM-1 intracellular domain is involved in flow-stimulated endocytosis of Ab/NC. We employed REN cells (which are naturally PECAM-1 null) transfected with full-length PECAM-1 vs mutant PECAM-1 lacking the cytosolic domain, as described in our previous work [18]. The results shown in Fig. 5A indicate that: i) flow does stimulate uptake of Ab_{37}/NC by non-endothelial REN cells expressing full-length PECAM-1, which is similar to our observation in endothelial cells and thus validates this model; and, ii) deletion of PECAM-1 cytosolic domain abrogates this effect of flow. Further, flow did not enhance endocytosis of either Ab_{62}/NC or Ab_{37}/NC (50 and 200 Ab per NC, respectively) in REN cells expressing phosphorylation deficiency mutant (Y686F) of PECAM-1 (Fig. 5B). Therefore, flow stimulates Ab/NC endocytosis via signaling pathway(s) involving cholesterol-rich domains of plasmalemma and Tyr686 in cytosolic tail of PECAM-1.

2.3. Stimulation of endothelial uptake of Ab_{62}/NC and Ab_{37}/NC by flow involves different intracellular signaling pathways

We addressed the role of RhoA/ROCK signaling pathway(s) and actin cytoskeleton rearrangements in modulation of Ab/NC internalization by flow. Treatment of endothelial cells with Y-27632, a pharmacological agent that inhibits RhoA/ROCK, decreased polymerized actin bundles in endothelial cells under static conditions, and prevented rearrangement of the endothelial actin cytoskeleton in response to flow (Fig. 6A), in accord with the literature establishing the pivotal role of

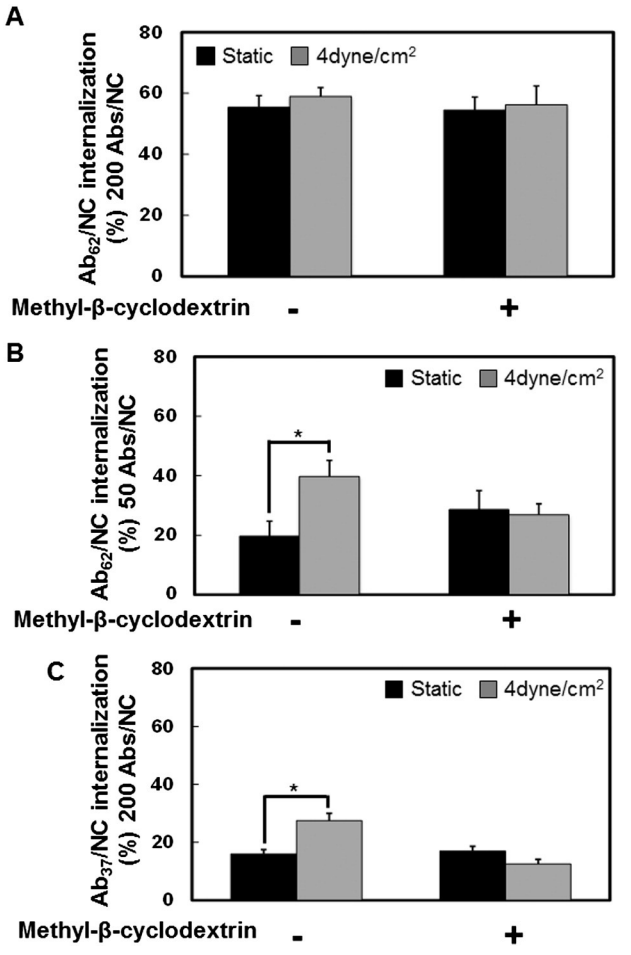

Fig. 4. Disruption of lipid rafts abolished the shear stress-stimulated endocytosis of Ab_{37}/NC and Ab_{62}/NC. Methyl-β-cyclodextrin pretreatment inhibited shear stress (4 dyn/cm^2)-induced endocytosis of Ab_{62}/NC (200 Abs/NC, *A*), (50 Abs/NC, *B*) and Ab_{37}/NC (200 Abs/NC, *C*). Confluent endothelial cells were pre-treated with Methyl-β-cyclodextrin (1 mM) for 30 min, followed by incubation or perfusion of Ab/NC for 30 min in the presence of Methyl-β-cyclodextrin. Internalization of Ab/NC was analyzed and expressed as Mean ± S.E. (n = 6, $^*p < 0.05$, in comparison with static groups).

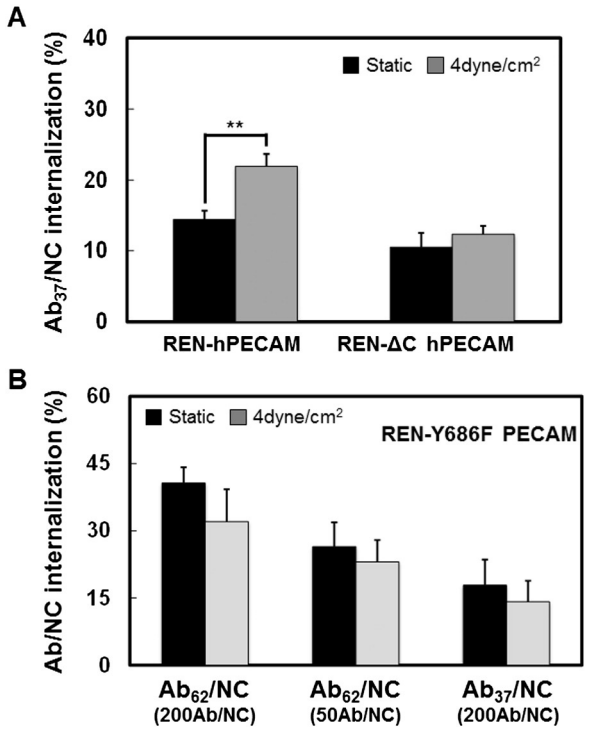

Fig. 5. Phosphorylation of PECAM-1 cytoplasmic domain is required for fluid shear stress-stimulated endocytosis of Ab/NC. *A*, Cytoplasmic domain of PECAM-1 mediated the stimulatory effect of flow (4 dyn/cm^2, 30 min) on endocytosis of Ab_{37}/NC. *B*, Expression of phospho-defective PECAM-1 mutant Y686F inhibited flow-stimulated endocytosis of Ab_{37}/NC (200 Ab/NC) and Ab_{62}/NC (50 Ab/NC). REN cells stably expressing human PECAM-1 WT and cytoplasmic domain deleted mutant ΔCD (*A*), or phosphor-defective mutant Y686F (*B*) were incubated or perfused with Ab_{37}/NC for 30 min at 37 °C. Internalization of Ab_{37}/NC and Ab_{62}/NC was analyzed and expressed as Mean ± S.E. (n = 6, $^{**}p < 0.01$).

RhoA/ROCK signaling in dynamic regulation of cytoskeleton in endothelial cells. Based on this, we examined the role of this signaling in regulation of endocytosis of Ab/NC by flow. We found that inhibition of RhoA/ROCK by Y-27632: i) suppressed internalization of high-avidity Ab_{62}/NC (200 Abs/NC) under both static and flow conditions (Fig. 6B); ii) abrogated flow-induced stimulation of uptake of low avidity Ab_{62}/NC (50 Abs/NC, Fig. 6C); and, iii) in stark contrast, did not affect the stimulatory effect of flow on Ab_{37}/NC uptake (Fig. 6D). This result suggests that flow stimulates endocytosis of Ab_{62}/NC and Ab_{37}/NC via different signaling mechanisms.

Finally, we tested the role of Src-family kinases (SFK). In a sharp contrast to RhoA/ROCK inhibition, pharmacological inhibition of SFKs by pretreatment of endothelial cells with PP2 had no effect on flow-induced stimulation of the uptake of Ab_{62}/NC, whereas it abrogated flow-induced stimulation of Ab_{37}/NC internalization (Fig. 7).

3. Discussion

PECAM-1, a cell adhesion molecule abundantly expressed in endothelial cells, supports adhesion and trans-endothelial migration of leukocytes in inflammation sites [28–30]. Among other endothelial surface determinants, PECAM-1 is emerging as an attractive target for endothelial drug delivery [23,31,32]. Drug-loaded NC targeted to PECAM-1 bind to the endothelium and exert therapeutic effects unattainable by non-targeted drugs in animal models [32–35]. Further, endocytosis induced by multivalent Ab/NC targeted to PECAM-1 enables intra-endothelial delivery of carriers and their cargos [21,22,36]. Understanding the factors controlling endothelial delivery of Ab/NC may help to optimize drug delivery and precise addressing at the subcellular level, further improving therapeutic potential of this prospective drug delivery system.

Previous studies revealed that endocytosis of Ab/NC is modulated by: i) microenvironment and physiological state of endothelium; ii) specificity of binding to distinct epitopes on PECAM-1; and, iii) Ab/NC configuration (e.g., antibody surface density). Present study extends these paradigms in the context of flow, an important factor of vascular physiology that influences all aspects of Ab/NC targeting including its delivery to, binding and internalization by endothelium.

In this context, it is important to consider targeting features of the PECAM-1 antibodies employed in the study and distinguish Ab/NC internalization from binding. The data of previous studies, summarized in Supplemental table 1, indicate that Ab_{62} and Ab_{37} bind to distinct epitopes in the most distal Ig-like domain 1 (IgD1) of the extracellular portion of human PECAM-1 [37]. Noteworthy, despite the fact that Ab_{37} has a higher endothelial affinity than Ab_{62} [38], Ab_{62}/NC bind to endothelial cells better than Ab_{37}/NC (Fig. 1A). This outcome implies that either: i) avidity of Ab_{62}/NC is higher (e.g., due to less damaging immobilization or more optimal antibody orientation on the particle surface); or/and, ii) the accessibility of Ab_{62} epitope for multivalent binding of large ligands is superior to that of Ab_{37}.

The defining specific aspects of binding and internalization are important, since Ab/NCs induce endocytosis by PECAM-1 cross-linking caused by multivalent binding. In theory, binding of more Ab/NC engaging more copies of PECAM-1 molecules per cell (attained by Ab_{62}/NC vs

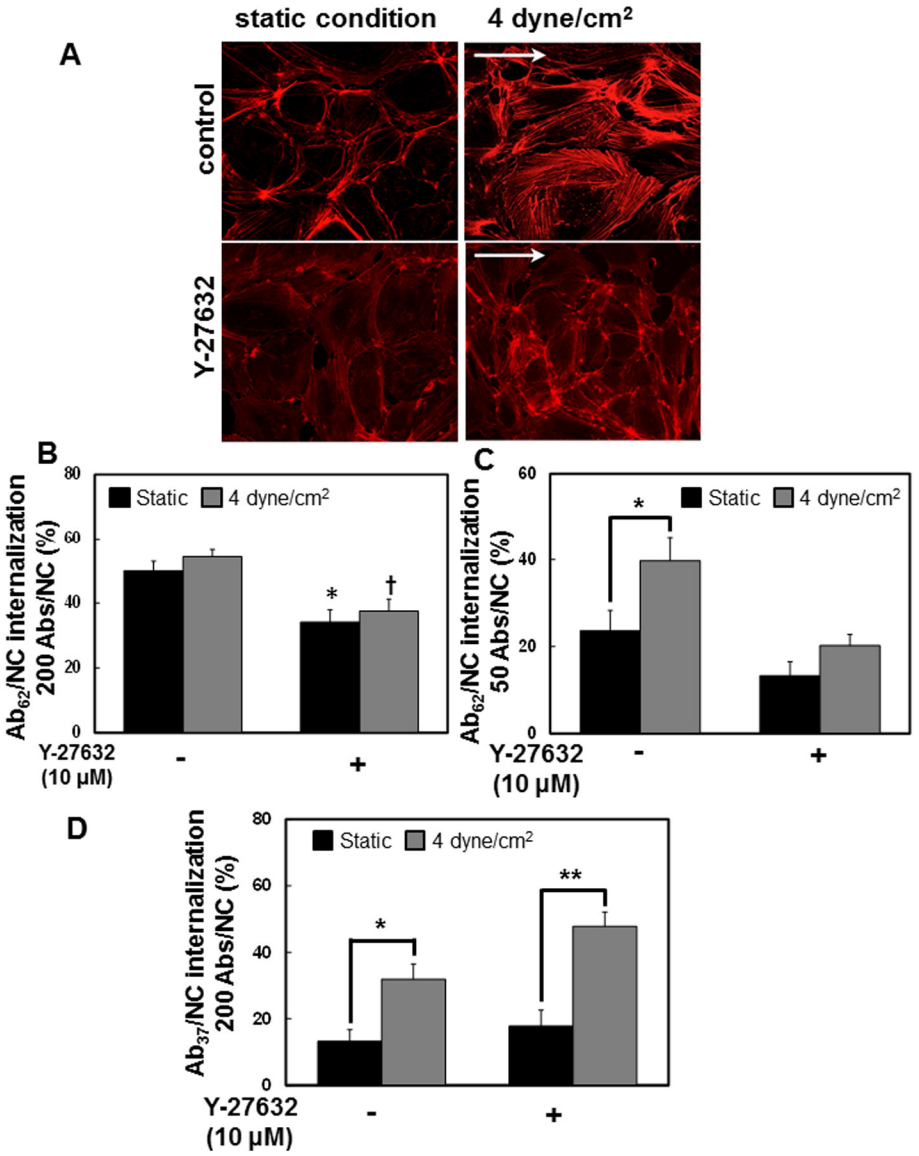

Fig. 6. Blockage of RhoA/ROCK signaling pathway specifically inhibits shear stress-induced endocytosis of Ab_{62}/NC. *A*, Y-27632, a Rho-kinase inhibitor, prevented cytoskeletal remodeling in response to flow dependent shear stress. Confluent endothelial cells were incubated or perfused at a flow of 4 dyn/cm^2 with culture medium in the absence or presence of Y-27632 (10 μM) for 30 min. Cells were then fixed and stained for F-actin using Alexa-Fluor594-phalloidin. Images were taken using fluorescence microscope with a Plan Apo 40× oil objective. Arrows show direction of flow. *B* and *C*, pretreatment of endothelial cells with Y-27632 (10 μM, 30 min) diminished the endocytosis of Ab_{62}/NC (200 Abs/NC (*A*), 50 Abs/NC (*B*)) under both static and flow conditions. *D*, Y-27632 pretreatment did not inhibit shear stress-stimulated endocytosis of Ab_{37}/NC (200 Abs/NC).*† $p < 0.05$, **$p < 0.01$ in comparison with static groups.

Ab_{37}/NC) may elicit stronger endocytic signal. However, data shown in Fig. 2 indicate that endocytosis of Ab/NC is modulated by strength of signaling from an individual Ab/NC anchored to PECAM-1, not the total number of cell-bound Ab/NC particles or the total number of PECAM-1 copies engaged. This squares well with previous observations for NC targeted to the CAM pathway [12,18,26].

Blood flow alters the adhesive interactions between Ab/NC and endothelium [27,39]. Flow-driven rolling on the endothelial surface may assist Ab/NC in engaging PECAM-1, thereby increasing the strength of CAM-endocytic signaling. Alternatively, rotational motion due to the flow-derived torque force applied to PECAM-1-anchored Ab/NCs may further mechanically stimulate endothelial cells and enhance signaling for Ab/NC internalization.

On the other hand, flow is known to modulate many parameters of the endothelial functional status, some of which may be involved in endocytic processes directly or indirectly. In fact, shear stress governs endothelial processes including cytoskeletal remodeling, gene expression, ion transport and endocytosis [40]. It has long been recognized that internalization of extracellular fluid and macromolecules such as LDL into endothelial cells is stimulated by flow [40,41]. Stimulatory effects of flow has been observed in endothelial pinocytosis [40,42], clathrin-dependent [41,42], and CAM-dependent endocytosis [12,18]. It is conceivable that flow stimulates endocytosis via a generalized mechanism, such as enhancement of the rate of plasmalemma vesicle maturation, or dynamic changes of the cytoskeleton.

However, one could also expect that at least some components of mechanisms of flow-sensitive modulation of endocytosis are specific for distinct types of endocytic processes and, perhaps, distinct receptors and ligands. For example, flow stimulates uptake of albumin in kidney proximal tubule cells via a clathrin-dependent endocytic pathway [42]. Findings presented in our study showed for the first time that distinct mechanisms mediate flow-stimulated endothelial endocytosis of Ab/NC targeted to different epitopes of PECAM-1.

Studies in static endothelial cells showed that PECAM-1 multivalent engagement by Ab/NCs activates signaling mediated by small GTPase RhoA/ROCK pathway, leading to rapid formation of actin stress fibers,

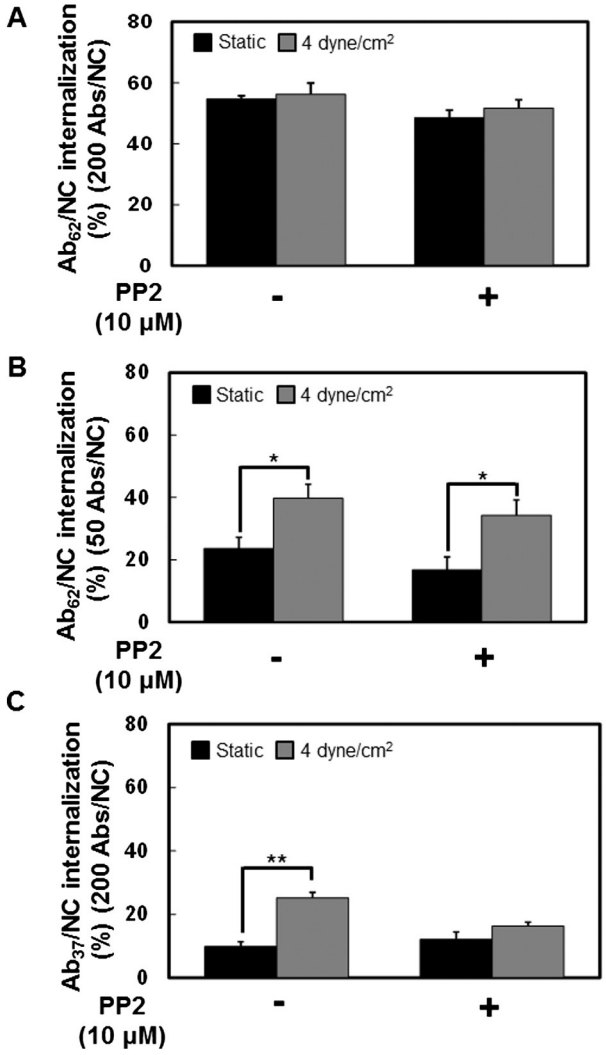

Fig. 7. Inhibition of Src family kinases specifically abolishes fluid shear stress-induced endocytosis of Ab_{37}/NC. *A* and *B*, pretreatment of endothelial cells with PP2 (10 μM), Src family kinases inhibitor, could not inhibit the endocytosis of Ab_{62}/NC (200 Abs/NC (*A*), 50 Abs/NC (*B*)) under either static or flow conditions. *C*, PP2 pretreatment inhibited shear stress-stimulated endocytosis of Ab_{37}/NC (200 Abs/NC).*$p < 0.05$, **$p < 0.01$ in comparison with static groups.

necessary for CAM-endocytosis [25,26]. In addition to "facultative" involvement in CAM-endocytosis serving "quasi-physiological" internalization of artificial objects such as Ab/NC, RhoA/ROCK is the key regulator of physiological dynamic actin rearrangements underlying endothelial responses to mechanical forces [43]. Therefore, rearrangements of actin cytoskeleton play a complex role both in Ab/NC uptake [12,18,44] and in cellular responses to shear stress [45]. On one hand, CAM-endocytosis requires recruitment of actin to the sites of Ab/NC binding and formation of stress fibers involved in vesicular uptake [46,47], on the other hand, RhoA-mediated signaling to cytoskeleton is required for endothelial functional responses to flow in the signaling chain components downstream of PECAM-1 [48]. Chronic flow exposure causes substantial commitment of actin to stable flow direction-oriented stress fibers thereby limiting actin recruitment to the endocytic pool and inhibiting CAM-endocytosis [12,18]. In contrast, transient exposure to flow stimulates CAM-endocytosis, perhaps by priming actin-rearrangement. The latter mechanism may be involved in the phenomena observed in the present study as well.

The complexity of mechanisms underlying Ab/NC endocytosis and its regulation by flow is emphasized by the fact that PECAM-1 also functions as an endothelial sensor for biomechanical stimuli including hemodynamic factors [49–51]. Thus, phosphorylation at Tyr686 residue in PECAM-1 cytosolic domain is the key event in both endothelial flow shear stress sensing [49,52] and Ab/NC internalization caused by engagement of the extracellular region under static condition [26,53].

Furthermore, in addition to RhoA/ROCK pathway, the Src family kinases (SFKs) signaling pathway is involved in both endothelial flow sensing and Ab/NC endocytosis. SFKs are signaling enzymes regulating cellular proliferation, survival, migration and metastasis [54,55]. In particular, flow causes SFK-dependent signaling in endothelial cells via PECAM-1 phosphorylation at Tyr686 [56–59]. Activation of SFKs is also involved in endocytic pathways, including caveolae- and CAM-mediated endocytosis [25,60,61]. Results shown in Figs. 6 and 7 indicate that stimulation of internalization of Ab_{62}/NC and Ab_{37}/NC involves alternatively RhoA/ROCK and SFK signaling pathways, respectively.

In addition to PECAM-1, specific microdomains of plasma membrane enriched in cholesterol and sphingolipids, i.e., lipid rafts and caveolae, function in endothelial cells as sensing and signaling platforms for mechanotransduction [62]. These microdomains are also involved in membrane trafficking and vesicular uptake and transport [63]. Signaling and endocytic pathways sensitive to cholesterol depletion include lipid rafts, caveolae and several forms of pinocytosis [62–67]. Numerous studies showed that Ab_{62}/NCs do not co-localize in caveolar markers during endocytosis under either static [25] or flow conditions [18]. Flow also has been shown to stimulate endothelial pinocytosis [40]. However, Ab/NC endocytosis via PECAM-1 is clearly distinct from this receptor-independent constitutive pathway for fluid-phase uptake [68]. Therefore, it is more plausible that cholesterol-rich membrane domains play indirect signaling rather than direct endocytic function in stimulation of Ab/NC internalization. Perhaps, auxiliary signaling via these domains in response to flow augments mechanosensing and endocytic functions mediated by PECAM-1. These signaling pathways are not yet fully understood but include the interplay between blood flow, cholesterol-rich plasmalemma domains, cytoskeleton rearrangements, Tyr686 phosphorylation of PECAM-1 and signaling via RhoA/ROCK and SFK (see a hypothetic schema in Fig. 8).

Understanding the molecular mechanisms and hemodynamic control of Ab/NC internalization is important for rational design of endothelial nanomedicine targeted to specific vascular areas, particularly in those regions where the endothelium is exposed to distinct flow conditions. The context of this paper is limited principally to unidirectional

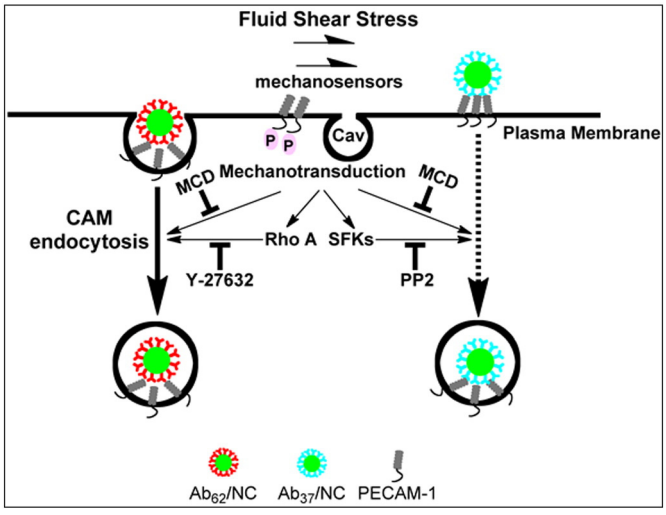

Fig. 8. Fluid shear stress stimulates the endocytosis of Ab_{37}/NC and Ab_{62}/NC via distinct mechanisms. Disruption of lipid rafts by methyl-β-cyclodextrin, a cholesterol chelator, abolishes acute shear stress-induced endocytosis of Ab_{62}/NC and Ab_{37}/NC. Blockage of RhoA/ROCK signaling pathway by Y-27632 selectively inhibits shear-stress-stimulated endocytosis of Ab_{62}/NC. Inhibition of Src family kinases (SFKs) by PP2 specifically blocks flow-stimulated endocytosis of Ab_{37}/NC.

flow typical of small blood vessels and the microcirculation [69], important large reservoirs of endothelial surface area for NC delivery. The *ibidi* system delivers levels of steady laminar shear stress that are non-pulsatile and are of low Reynolds numbers (Re) — the ratio of inertial forces to viscous forces that quantifies the relative importance of these two types of forces for given flow conditions. In contrast, Re in large arteries are much higher (Re: several hundred to > 2000) than in small arterioles and capillaries (Re: < 1.0) at comparable wall shear stress values. These differences potentially have profound implications for local transport rates at the endothelial surface, an element of obvious importance to NC delivery. Exploration of NC behavior in a high Re model of arterial flow using a large volume, pulsatile fast flow system [70] will investigate complex separations of flow (eddies, vortices) typical of athero-susceptible arterial sites [71] and is beyond the scope of the present report.

It would be somewhat naive to attempt to translate our findings into a guidance which type of PECAM-1 ligands (e.g., Ab_{37} or Ab_{62}) will provide optimal internalization in a vascular area of interest based on the hydrodynamic factors typical of that area. Our knowledge of mechanisms of these processes and ability to determine hydrodynamic characteristics in the patient's vessels (especially those with complex branching and meandering configuration, or/and affected by pathological process) are quite limited. However, our study indicates for the first time that, in theory, such a rational design of epitope-specific intracellular delivery governed by flow is possible. Further, it noteworthy for the drug delivery field that nearly identical carriers binding to the adjacent epitopes on the anchoring molecule may have different targeting features differently modulated by local biological factors including biomechanical conditions.

4. Conclusion and perspectives

CAM-endocytosis offers targeted delivery of NCs into endothelial cells. Our results indicate that flow modulates endothelial endocytosis of Ab/NC mediated by PECAM-1 in an epitope-specific manner, via mechanisms involving complex and differential signaling pathways (Fig. 8). The notion that flow modulates endocytosis in a PECAM-1-epitope specific manner and within a certain range of Ab/NC avidities is important in the context of selecting optimal affinity ligands and devising their configuration into drug delivery systems which need to induce intracellular transport while exposed to the circulation.

5. Methods

5.1. Materials

Monoclonal antibodies to human PECAM-1 (anti-PECAM) were Ab_{62} and Ab_{37}, kindly provided by Dr. Marian Nakada (Centocor) [23]. Fluorescent secondary antibodies were from Invitrogen (Carlsbad, CA). Fluorescein isothiocyanate (FITC) labeled polystyrene spheres (100 nm in diameter) were purchased from Polysciences (Warrington, PA). Methyl-β-cyclodextrin, Y-27632 and PP2 were obtained from Sigma (St. Louis, MO).

5.2. Preparation of anti-PECAM-1/NC

FITC-labeled polystyrene spheres were coated with either anti-PECAM-1 antibodies (Ab_{62} or Ab_{37}) or control murine IgG via incubation at room temperature (RT) for 1 h [25]. The reaction mixture was centrifuged to remove unbound materials, then re-suspended in 1% bovine serum albumin (BSA)-PBS and microsonicated for 20 s at low power. The effective immunobead diameter was determined by dynamic light scattering (DLS) using a BI-90 Plus particle size analyzer with BI-9000AT Digital auto-correlator (Brookhaven Instruments, Brookhaven, NY). This protocol yields uniform preparations of anti-PECAM Ab/NC with particle diameters ranging from 180 to 220 nm, indicated thereafter as Ab_{62}/NC and Ab_{37}/NC unless specified otherwise. The saturating antibody surface coverage on the NC surface was estimated to be ~200 antibody molecules per particle [72]. To prepare Ab/NC with variable antibody surface densities (50 and 200 anti-PECAM molecules per NC), the polystyrene spheres were coated with a mix of anti-PECAM antibody and IgG at molar ratios 1:3, 1:0, respectively, keeping the total amount of IgG molecules coated per particle (including anti-PECAM Ab and IgG) constant to avoid variability due to different surface coatings [72].

5.3. Cell culture and treatments

Endothelial cells used in these studies were human umbilical vein endothelial cells, purchased at passage 1 from Lonza (Walkersville, MD) and cultured for up to six passages in endothelial basal medium (EBM-2) supplemented with EGM-2 Single Quote (Lonza). Cells were starved overnight in EBM-2 containing 0.5% fetal bovine serum without supplements prior to experiments. Anti-PECAM1 Ab or IgG coated nanocarriers were then added to HBSS in the reservoir to the final carrier concentration of 2.0×10^9 carriers/ml unless specified otherwise, and were perfused for 30 min. Thereafter, cells were washed extensively with HBSS to remove unbound particles prior to fixation with 1% paraformaldehyde for fluorescent staining and analysis.

In the experiments to examine the effects of Methyl-β-cyclodextrin, Y-27632 and PP2 on endocytosis of Ab_{62}/NC and Ab_{37}/NC, endothelial cells were pre-incubated with Methyl-β-cyclodextrin (1 mM), Y-27632 (10 μM) and PP2 (10 μM) for 30 min. Thereafter, cells were incubated or perfused with anti-PECAM1 Ab NCs in the presence of Methyl-β-cyclodextrin, Y-27632 and PP2 for 30 min.

The human mesothelioma REN cells stably expressing human wild-type PECAM-1 or cytoplasmic domain deleted mutant ΔPECAM-1 used in this study have been previously described [26]. REN cells were cultured in RPMI1640 supplemented with 10% FBS and Geneticin (G418) as a selection agent.

5.4. In vitro laminar shear stress system

A six-channel μ-slide flow chamber (Ibidi, Germany) was used to subject endothelial monolayers to defined laminar shear stress. The chamber was connected to a recirculating flow circuit composed of a variable-speed peristaltic pump (Rainin RP-1, Columbus,OH), a reservoir with culture medium, and inlet and outlet silicone rubber tubing. The flow rate was calibrated by collecting the volume of medium discharged per minute. The wall shear stress generated by fully-developed fluid flow through the channel was calculated using $\tau = 6\mu Q/h^3 w$, where μ is the fluid viscosity (μ = 0.70 cP for HBSS at 37 °C), Q is the mean velocity of the flow through the channel, and H and W are the channel height (0.4 mm) and width (3.8 mm), respectively. Shear stresses were in the range 0–8 dyn/cm^2. The temperature was maintained at 37 °C, and pH and gases were maintained in a 95% air/ 5% CO_2 incubation chamber.

5.5. Microscopy and quantification of cell-bound and internalized Ab/NC

Endothelial monolayers or REN cells were washed with Hank's balanced salt solution (HBSS) to remove unbound NC following endothelial uptake of Ab/NC. Cells were then fixed with 1% paraformaldehyde for 10 min. To distinguish between surface-bound or internalized immunobeads, non-permeabilized fixed cells were counterstained for 30 min at RT with Alexa-Fluor-594-conjugated goat anti-mouse IgG to produce double-labeled, yellow particles. The cells were washed five times with HBSS containing 0.05% Tween-20, mounted with ProLong Antifade Kit (Molecular probes, Eugene OR) and analyzed by fluorescence microscopy.

Endothelial cells grown in the flow chamber were exposed to flow (4 dyn/cm^2) for 30 min. Cells were fixed, permeabilized with 0.1% Triton

X-100 for 15 min, and stained for F-actin (stress fibers) with Alexa-Fluor-594-phalloidin (Molecular probes, Eugene, OR).

Fluorescence microscopy was performed with an Olympus IX70 inverted fluorescence microscope, 40× PlanApo objectives and filters optimized for green fluorescence (excitation BP460-490 nm, dichroic DM570 nm, emission BA515-550 nm) and red fluorescence (excitation BP530-550 nm, dichroic DM570 nm, emission BA590-800 +). Separate images for each fluorescence channel were acquired using a Hamamatsu Orca-1 CCD camera. The images in green and red channel were merged and analyzed with ImagePro 3.0 imaging software (Media Cybernetics, Silver Spring, MD). Green and red fluorescence images were separately obtained by means of gain and exposure times that were optimized to produce 8-bit images with average background intensity values of approximately 20 bits per pixel and average maximum intensity values of approximately 250 bits per pixel (below saturation). Once the settings were established, they were used for all images obtained for a given sample. For particle quantification, double-labeled particles showing yellow color were identified as surface bound immunobeads by generating a new RBG image merging the green and red channels, and were scored using ImageJ particle analyze plugin with the constraint that only regions with 4 or more continuous pixels and with an intensity threshold of 128 were counted. The green fluorescence image was then scored in a compatible manner to give the total number of immunobeads in the field. Endocytosis was calculated as the percentage of internalized immunobeads with respect to the total number of cell-associated immunobeads. The data are shown as means from ≥6 images ± S.E. Statistical significance between groups was determined by Student's t test and was accepted as significant at $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.05.006.

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