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Talin-2 regulates integrin functions in exosomes

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ABSTRACT

Integrins on exosomes have been shown to mediate binding to recipient cells, potentially playing important roles in controlling exosomal internalization and organ distributions. Although the ability of cellular integrins to mediate cell adhesion is known to be regulated by the cytoplasmic adaptor protein talin, whether the activity of exosomal integrins is similarly regulated by talin remains to be elucidated. Here we have studied this question in T-cell exosomes that surface express the integrins $\alpha L\beta 2$ and $\alpha 4\beta 7$. T-cells and T-cell exosomes engineered to lack talin-2 showed reduced binding to the integrin ligand ICAM-1 and MAdCAM-1 compared with control T-cells and exosomes, despite the fact that those T cells and exosomes express intact levels of the other isoform talin-1. In addition, talin-2-deficient T-cell exosomes were less efficiently internalized by endothelial cells, compared with control exosomes. These results suggest that the mechanisms of talin-mediated integrin regulation operate similarly in cells and exosomes.

1. Introduction

Integrins are the family of $\alpha\beta$ heterodimeric cell adhesion molecules that mediate cell-to-cell and cell-to-extracellular matrix interactions across a wide range of biological phenomenon such as inflammation, thrombosis formation, cancer metastasis, wound healing and organ development [1,2]. By binding to their cognate ligands expressed on the opposing cells and/or present in extracellular matrices, integrins regulate cellular localization, migration and trafficking in health and diseases [3].

Integrin $\alpha L\beta 2$ or lymphocyte function-associated antigen-1 (LFA-1) is the major adhesion receptor expressed in all lymphocytes, and it mediates a shear-resistant adhesive interaction with intercellular adhesion molecule-1 (ICAM-1) on endothelial cells [4]. Integrin $\alpha 4\beta 7$ or lymphocyte Peyer's patch adhesion molecules-1 (LPAM-1) is expressed in a subset of lymphocytes, in which it supports adhesive interactions with its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed in gut endothelial cells [5]. In cooperation with integrin $\alpha L\beta 2$, integrin $\alpha 4\beta 7$ enables lymphocyte trafficking specifically to gut mucosal tissues [6]. The upregulation of integrin $\alpha 4\beta 7$ expression marks gut-tropic T-lymphocyte subsets [7]. Integrin $\alpha 4\beta 7$-mediated lymphocyte trafficking to the gut plays an important role both in the regulation of the mucosal immune system [6], and in the pathogenesis of the sustained mucosal inflammation observed in inflammatory bowel diseases [3]. We have recently shown that gut-tropic T-lymphocytes secrete exosomes, the lipid-bilayer biological nanoparticles involved in intercellular communications, that express high levels of integrin $\alpha 4\beta 7$ [8]. Integrin $\alpha 4\beta 7^{high}$ T-cell exosomes are preferentially distributed to the gut, wherein they down-regulate MAdCAM-1 expression in gut endothelial cells via exosomal microRNAs. Thus, integrin $\alpha 4\beta 7^{high}$ T-cell exosomes are believed to represent the negative regulatory mechanism that would down-size excessive T-cell trafficking to the gut [8].

The ability of integrins to bind ligands is regulated by intracellular signaling cascades that culminate in the association of adaptor proteins with cytoplasmic integrin domains [9]. Talin is the major adaptor protein that acts as “a final common step in integrin activation” [10]. Talin becomes associated with the integrin β cytoplasmic domain, thereby triggering the conformational changes of

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the integrin ectodomains to the ligand-competent high-affinity form [10–12]. After integrins bind their ligands, talin is still required for stabilizing the ligand-bound high-affinity form of integrins [13]. In this way, talin plays critical roles in regulating many aspects of integrin-mediated adhesive interactions.

In cells, talin has been demonstrated to regulate the activity of integrin $\alpha L\beta 2$ [11] and $\alpha 4\beta 7$ [14] to bind ligands. However, it remains to be elucidated whether the activity of exosomal integrins is regulated by exosomal talin. This question is of great biological importance, since exosomal integrins play important roles both in the tissue distribution of exosomes and in the uptake of exosomes by recipient cells. To address this question, we studied T-cell exosomes lacking talin-2, one of two talin isoforms (i.e., talin-1 and talin-2). We have shown that talin-2 null T-cell exosomes exhibited a reduced ability to bind to MAdCAM-1 and ICAM-1, compared with talin-2 intact T-cell exosomes, despite the intact expression of exosomal talin-1. Furthermore, talin-2 null T-cell exosomes were less efficiently taken up by endothelial cells expressing MAdCAM-1 and ICAM-1. These results have demonstrated the critical roles of talin-2 in integrin functions in exosomes.

2. Methods

2.1. Reagents and antibodies

The reagents and antibodies used in this study are described in Supplemental Information.

2.2. Cell culture

TK1 cells (mouse T cell line) and bEnd.3 cells (mouse endothelial cell line) were obtained from the American Type Culture Collection and were cultured as previously described [8].

2.3. Talin-2 knockout cells

The methods used to generate talin-2 knockout cells are described in Supplemental Information [15–17].

2.4. Isolation of exosomes

Exosomes from the culture supernatants of control and KO TK1 cells were isolated via differential ultracentrifugation (UC) as previously described [8,18].

2.5. Nanoparticle tracking analysis (NTA)

The size distribution of the exosomes released from TK1 cells was determined via NTA using NanoSight LM10 (Malvern Instruments, UK) as previously described [8,19].

2.6. Western blot

Western blot analysis was performed as previously described [8].

2.7. Flow cytometry analysis of exosomes

Exosomes were conjugated to the surface of latex beads as previously described [8,20]. The exosome-conjugated beads were probed with primary antibodies diluted in PBS/0.5% BSA and the analysis was conducted with BD Accuri C6 plus flow cytometer (BD Biosciences, San Jose, CA) as previously described [8].

2.8. Adhesion assay for cells and exosomes-coated microspheres

Adhesion assays were performed as previously described [21]. TK1 cells were fluorescently labeled with 1 mM BCECF-AM (Dojindo, Japan). In the case of exosomes, 10 μg of exosomes were passively adsorbed to 10 μl of fluoresbrite plain microspheres (2.5% Solids-latex, 3 μm YG, Funakoshi, Japan catalog # 17155) as previously described [8]. Wells of a 96-well V-bottom plate were coated with MAdCAM-1-Fc or ICAM-1-Fc, as previously described [8]. Equal numbers of control and KO cells or an equal volume of exosome-coated microspheres were added to the wells in the presence of 1 mM magnesium chloride/calcium chloride (MgCl2/CaCl2) or 2 mM ethylene diamine tetra-acetic acid (EDTA). Samples in the presence of EDTA were used for determining background adhesion, as integrin-mediated interactions require divalent cations such as Mg2+ [8,21]. After incubation for 20 min at room temperature, the plates were centrifuged for 5 min at 1000 rpm for cells and 3000 rpm for exosome-coated microspheres. The amounts of non-adhered cells or non-adhered microspheres accumulated at the nadir of the V-bottom wells were quantified by 2030 ARVO X-2 Multilabel Reader (PerkinElmer, Japan). The percentage of bound cells or microspheres was calculated as previously described [21].

2.9. Uptake of fluorescently labeled exosomes by recipient cells

Exosomal uptake by bEnd.3 endothelial cells was studied as previously described [22]. bEnd.3 cells (1×10^5 cells) in DMEM medium with 10% exosome-free FBS were seeded into chambers of BD Falcon CultureSlides (BD Biosciences, San Jose, CA) and cultured for 48 h in the presence of 5 ng/ml TNF-α. Forty micrograms of control exosomes or KO exosomes resuspended in 200 μl of PBS were stained with Exo-Green Exosome Protein Fluorescent Label (System Biosciences, Palo Alto, CA) according to the manufacturer's protocol. bEnd.3 cells were then serum-starved and incubated with fluorescently labeled control exosomes, KO exosomes, or PBS as a vehicle control. The exosomes taken up by bEnd.3 cells were analyzed at 3-, 6- and 24-hr time points. At each time point, the culture slide was fixed with 2% paraformaldehyde for 15 min, stained with NucBlue Fixed Cell Stain (Thermo Fisher Scientific, Waltham, MA; catalog # R37606) at room temperature for 4 min, and then mounted with aqueous mounting medium (Abcam, Cambridge, UK; catalog # ab128982). Subsequently, the number of cells and exosome dots in each microscopic field was quantitated using a Fluorescence Microscope BZ-X700 (Keyence, Itasca, IL).

2.10. Statistical analysis

All data are presented as the mean ± SEM (standard errors of the mean). The comparison between two different groups was evaluated with a Student's *t*-test (unpaired, two-tailed). The resulting p values are indicated as follows: *, $0.01 < p < 0.05$, **, $0.001 < p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. Talin-2 deletion suppressed the integrin-mediated cell adhesion of TK1 cells

To investigate how talin-2 regulates integrin functions in T-cell exosomes, the mouse T-cell line TK1 was used as a model for the source of T-cell exosomes. TK1 cells, which expresses $\alpha 4\beta 7$ and $\alpha L\beta 2$ integrins, have been used for studying integrin functions in cells [23] and exosomes [8]. Using CRISPR/Cas9-mediated gene editing, we created talin-2 knockout (KO) TK1 cell clones and

corresponding vector-transduced control TK1 cell clones. We confirmed the deletion of talin-2 protein in talin-2 KO clones using Western blot analysis (Fig. 1A, left 3 lanes). The Western blot confirmed not only that TK1 cells express two isoforms of talin, namely talin-1 and talin-2, but also that the talin-2 KO clones show an intact expression of talin-1 protein comparable to that of the control clones. Thus, in the following sections, we addressed whether and how a talin-2 deletion would affect integrin functions in cells and exosomes in the presence of talin-1.

First, we investigated the impact of talin-2 deletion on integrin functions at the cell level in TK1 cells. An immunofluorescent flow cytometry analysis showed that the cell-surface expression of integrins $\alpha 4\beta 7$ and $\alpha L\beta 2$ were comparable between KO and control cells (Fig. 2, upper panels). Second, we examined the ability of integrins to bind ligand using a cell adhesion assay. We found that talin-2 KO TK1 cells exhibited a reduced binding to the $\alpha 4\beta 7$ integrin ligand MAdCAM-1 (Fig. 3A) and the $\alpha L\beta 2$ integrin ligand ICAM-1 (Fig. 3B). These results demonstrated that talin-2 plays indispensable roles in supporting integrin-mediated ligand binding in cells.

3.2. Integrin-mediated ligand binding is suppressed in talin-2 KO exosomes

To investigate the roles of talin-2 in regulating integrin functions at the exosome level, we isolated exosomes from the culture supernatants of talin-2 KO and control TK1 cells using ultracentrifugation. Western blot analysis confirmed the absence of talin-2 protein in exosomes secreted from talin-2 KO TK1 cells (Fig. 1, right 3 lanes). Talin-2 KO TK1 exosomes and control TK1 exosomes exhibited comparable expression of talin-1 (Fig. 3, right 3 lanes) and of an exosomal marker Alix protein as shown by Western blot analysis. In addition nano-tracking analysis demonstrated that both exosomes showed similar particle sizes (Fig. 1B).

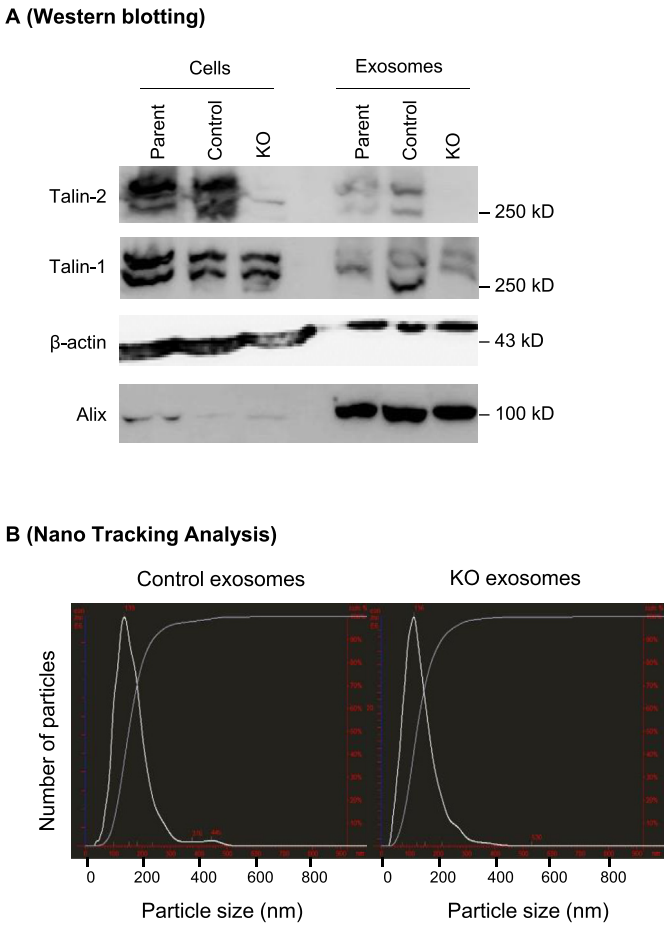

Fig. 1. Characterization of KO TK1 cells and exosomes. (A) Western blot analysis of cells (left 3 lanes) and exosomes (right 3 lanes) (**B**) Nano-tracking analysis of control (left) and talin-2 KO (right) TK1 exosomes.

To investigate the ability of exosomal integrins to bind their ligands, we performed a bead-based binding assay, in which microspheres coated with exosomes were allowed to interact with ligand substrates under the detachment force generated by low-speed centrifugation. We found that the binding of talin-2 KO exosomes to MAdCAM-1 and ICAM-1 was diminished, compared to binding of control exosomes (Fig. 4A and B). Thus, these results support the contention that talin-2 plays an essential role in exosomal integrin-mediated ligand binding, independently of talin-1 in exosomes.

3.3. Uptake of TK-1 exosomes by endothelial cells

The underlying mechanisms by which exosomes are taken up by recipient cells remain incompletely understood [24,25]. Exosomal integrins are thought to be involved in the receptor-mediated internalization of exosomes [25–27]. Thus, we sought to investigate the roles of talin-2-mediated regulation of exosomal integrin functions in the uptake of exosomes by recipient cells. As endothelial cells represent an important recipient cell of T-cell exosomes [8], we investigated how talin-2 affects the internalization of TK1 exosomes by endothelial cells. We used a mouse endothelial cell-line b.End3 that expresses both MAdCAM-1 and ICAM-1 and that has been used as a recipient cell model [8]. The b.End3 cells were incubated with equal amounts of fluorescently-labeled Talin-2 KO exosomes or control exosomes, and the levels of exosomal internalization were examined semi-quantitatively using fluorescent microscopy.

The uptake of KO and control exosomes by the endothelial cells gradually increased during the course of our observation up to 24 h (Fig. 4C). Notably, the uptake of KO exosomes by endothelial cells was less than that of control exosomes at all time points tested (3, 6, and 24 h after the addition of exosomes). These results support that idea that talin-2 mediated exosomal integrin binding to ligands on target cells could be an important factor for the uptake of exosomes.

4. Discussion

The activity of integrins on the cell surface is known to be regulated by the cytoplasmic adaptor protein talin [10–12]; however, whether such activity is similarly regulated in the surface of exosomes remains to be elucidated. The present study has addressed this previously unsolved question regarding the roles of talin in regulating the integrin functions in exosomes by specifically knocking out talin-2 in exosomes. The results have demonstrated that the activities of the exosomal integrins α4β7 and αLβ2 to bind ligands MAdCAM-1 and ICAM-1, respectively, are regulated by talin-2. In the absence of talin-2, integrin-mediated ligand binding was reduced not only in T cells, but also in T-cell exosomes, despite the presence of intact talin-1 protein. Thus, these results support the contention that the mechanisms by which integrin activity is upregulated through cytoplasmic interactions with talin is conserved in exosomes. Furthermore, this study has implicated talin-2-regulated exosomal integrin activity in facilitating the process of exosomal uptake by endothelial cells that express MAdCAM-1 and ICAM-1, as talin-2 KO exosomes were less well

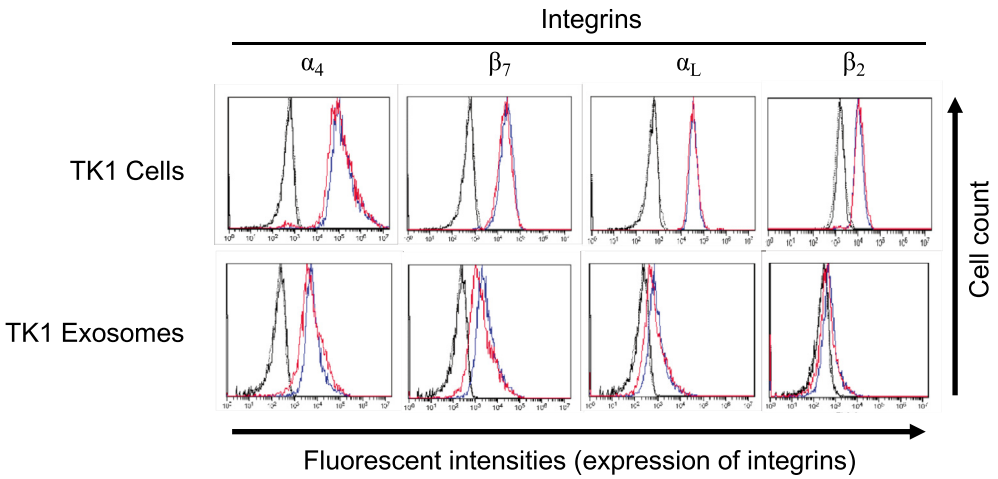

Fig. 2. Flow cytometric analysis of integrin expression. (Top) Representative FACS histograms showing the expression of integrins on talin-2 KO (red) and control (blue) TK1 cells. A background staining with isotype control antibodies is shown in black lines (dot, KO; solid, control). **(Bottom)** Representative FACS histograms showing the expression of integrins on talin-2 KO (red) and control (blue) TK1 exosomes coated on microspheres. In all panels, a background staining with isotype control antibodies is shown in black lines (dot, KO; solid, control). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

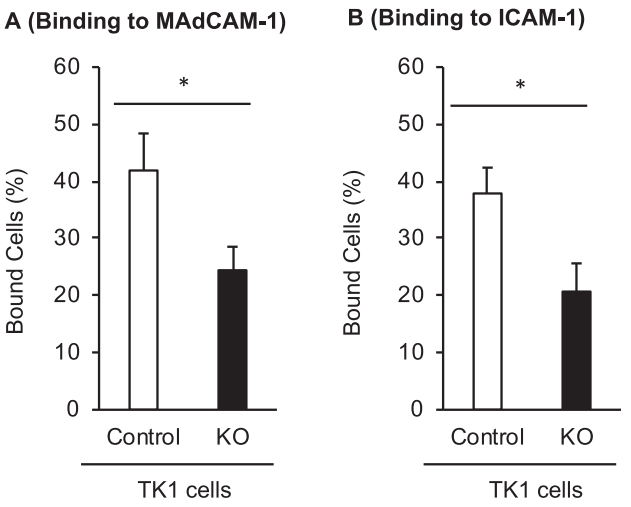

Fig. 3. Integrin-mediated cell adhesion of talin-2 KO and control TK1 cells. Cell adhesion of talin-2 KO and control TK1 cells to MAdCAM-1 **(A)** and ICAM-1 **(B)** substrates was quantitatively examined. Results are expressed as the mean ± SEM of three independent experiments. *0.01 < p < 0.05 between KO and control.

internalized by endothelial cells.

A previous series of structural and functional studies established the mechanistic basis of talin-mediated upregulation of integrin activities to bind ligands. The binding of talin to the integrin cytoplasmic domain induces the ligand-competent high-affinity conformation of the integrin ectodomains, a process termed inside-out signaling [1,2]. Conversely, the binding of cell-surface ligands to the integrin ectodomains stabilizes the high-affinity conformation, thereby facilitating the cytoplasmic association with talin, which is known as outside-in signaling [1,2]. In this way, the bi-directional (i.e., inside-out and outside-in) transmembrane signaling involving the interactions between, talin, integrins, and ligands represents a central mechanism for regulating integrin-mediated cell adhesion to opposing cells, as well as to the extracellular matrix [1,2]. This study strongly supports the idea that a similar mechanism operates in the regulation of exosomal integrins, although the direct physical association of integrins and talin in exosomes remains to be demonstrated. As experimentally demonstrating the association of integrins with talin has proven technically challenging in cells [11,28], future investigations are needed to address this question in exosomes.

Two isoforms of talin, talin-1 and talin-2 proteins, which possess 74% of the amino acid sequence identity [29], have shown isoform-specific roles in some integrin functions [30,31], while exhibiting redundant roles in others [32]. Fibroblasts derived from talin-1 KO embryonic stem cells spread and adhered normally on a β1 integrin ligand fibronectin substrate, due to the presence of talin-2, the expression of which was increased in a compensatory mechanism [32]. By contrast, in the integrin-mediated mechanosensing at the focal adhesion, talin-1 and talin-2 have been shown to play isoform-specific roles [30]. In addition, it has been shown that talin-2 binds much more strongly to the integrin cytoplasmic domains than talin-1, thereby underscoring its indispensable role in generating traction force during cell migration [31]. The results in the present study that utilized talin-2 KO TK1 cells and exosomes demonstrated the indispensable role of talin-2 in regulating the activity of the cellular and exosomal integrins α4β7 and αLβ2. As talin-2 KO TK-1 cells and exosomes showed reduced, but residual, integrin-mediated ligand binding, those residual integrin activities are likely to be mediated via talin-1. Although investigations using talin-1 and talin-2 double KO cells and exosomes would be of interest, we were unable to generate such double KO cells due to technical problems (e.g., inability of double KO cells to proliferate).

Exosomes secreted to the extracellular space are eventually taken up by recipient cells, by which they deliver any exosomal contents, including small molecules and small RNAs, to the cytoplasm. Fusion to the cellular plasma membrane and endocytosis have been proposed as two major mechanisms of exosomal uptake [24,33]. Although exosomal integrins are thought to be involved in the integrin-ligand receptor-mediated endocytosis of exosomes [25–27], how the ligand-binding activity of exosomal integrins could modulate this process remained unclear. Our results that talin-2 KO TK1 exosomes were less well taken up by endothelial cells can likely be explained by the reduced ability of the exosomal integrins to bind MAdCAM-1 and ICAM-1 on endothelial cells. As MAdCAM-1 and ICAM-1 on endothelial cells are thought to internalize via endocytosis [34,35], it is possible that TK1 exosomes displaying α4β7 and αLβ2 integrins become bound to, and internalized through, endothelial MAdCAM-1 and ICAM-1. Talin-2 mediated upregulation of exosomal binding to MAdCAM-1 and

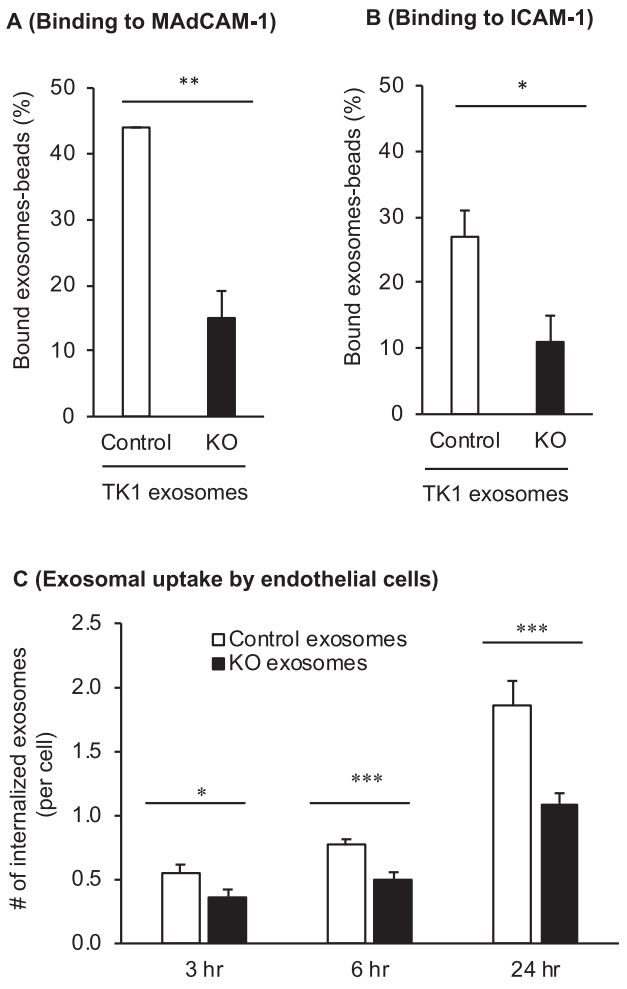

Fig. 4. Functional characterization of talin-2 KO and control TK1 exosomes. (**A** & **B**) Binding of talin-2 KO and control TK1 exosomes to MAdCAM-1 (**A**) and ICAM-1 (**B**), as examined by an adhesion assay using exosomes-coated microspheres. (**C**) Uptake of fluorescently-labeled talin-2 KO and control TK1 exosomes by endothelial cells, as quantitatively examined by fluorescent imaging. *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; and ***, $p < 0.001$.

ICAM-1 should increase not only the association of exosomes to endothelial cells, but also the subsequent internalization of exosomes by endothelial cells.

Exosomal integrins have been shown to determine the tissue-distributions of exosomes. The exosomal integrins α6β4 and αVβ5 preferentially guide cancer exosomes to laminin-expressing lung and fibronectin-expressing liver tissues, respectively [36]. We have previously shown that exosomal integrin α4β7 preferentially guides T-cell exosomes to MAdCAM-1-expressing high endothelial venules (HEVs) in the small intestine [8]. The mechanism by which talin upregulates the activities of integrins is thought to be conserved across different integrins. Thus, understanding how the tissue distributions of cancer and T-cell exosomes are regulated by talin would be of great importance, and therefore strongly warrants further investigations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.027.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.027.

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