Mechanisms and modulation of microvesicle uptake in a model of alveolar cell communication

Received for publication, April 20, 2017, and in revised form, October 27, 2017 Published, Papers in Press, November 3, 2017, DOI 10.1074/jbc.M117.792416

Daniel J. Schneider1, Jennifer M. Speth2, Loka R. Penke3, Scott H. Wettlaufer2, Joel A. Swanson4, and Marc Peters-Golden5

From the 1 Division of Pulmonary and Critical Care Medicine, 2 Department of Microbiology and Immunology, and 5 Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Edited by Thomas Söllner

Extracellular vesicles, including exosomes and shed microvesicles (MVs), can be internalized by recipient cells to modulate function. Although the mechanism by which extracellular vesicles are internalized is incompletely characterized, it is generally considered to involve endocytosis and an initial surface-binding event. Furthermore, modulation of uptake by microenvironment factors is largely unstudied. Here, we used flow cytometry, confocal microscopy, and pharmacologic and molecular targeting to address these gaps in knowledge in a model of pulmonary alveolar cell–cell communication. Alveolar macrophage–derived MVs were fully internalized by alveolar epithelial cells in a time-, dose-, and temperature-dependent manner. Uptake was dependent on dynamin and actin polymerization. However, it was neither saturable nor dependent on clathrin or receptor binding. Internalization was enhanced by extracellular proteins but was inhibited by cigarette smoke extract via oxidative disruption of actin polymerization. We conclude that MV internalization occurs via a pathway more consistent with fluid-phase than receptor-dependent endocytosis and is subject to bidirectional modulation by relevant pathologic perturbations.

Maintaining homeostasis in the face of perturbations demands a tightly regulated host response dependent on cell-cell communication. Traditionally, this is achieved by direct cell-cell contact and secretion of soluble mediators. An expanding body of research from various disciplines highlights extracellular vesicles (EVs) as additional vectors of cell-cell communication (reviewed in Ref. 1). EVs comprise two distinct types of submicron signaling packages surrounded by a lipid membrane, which are secreted from all cells: 1) exosomes (50–150-nm vesicles derived from multivesicular bodies) and 2) microvesicles (MVs)/microparticles (150–1000-nm vesicles generated by direct plasma membrane budding). Both harbor a variety of biologically active cargo including lipids, RNA, DNA, and soluble and surface proteins (reviewed in Ref. 2). EVs can act locally or systemically to alter function in recipient cells through surface interactions or internalization and intracellular cargo delivery. They have been implicated in homeostasis (reviewed in Ref. 3) and disease (reviewed in Ref. 4), and interest in their potential use as diagnostic markers and therapeutic vehicles is growing (5) (reviewed in Ref. 6).

Although the processes of EV secretion are reasonably well-studied, mechanisms governing their uptake have received substantially less attention. The vast majority of studies on uptake have focused on exosomes (reviewed in Ref. 7) rather than MVs. Moreover, many rely exclusively on qualitative analysis or on surrogate measures of uptake, such as functional changes in target cells. Whereas a minority of studies demonstrate membrane fusion of EVs (8–10), endocytosis is the predominant reported internalization mechanism (reviewed in Refs. 6 and 7). How similar these mechanisms are to classic endocytosis processes, such as receptor-dependent and fluid-phase endocytosis, is unclear. Moreover, it is virtually unknown how changes in the cellular microenvironment influence EV uptake.

Few microenvironments are subject to such a number and range of perturbations as is the alveolar surface of the lung. Recently, our laboratory demonstrated a novel form of EV-mediated intercellular communication regulating inflammatory signaling in the lung (11). Alveolar macrophages (AMs) were found to secrete suppressor of cytokine signaling 3 (SOCS3) protein within MVs, which in turn were taken up by alveolar epithelial cells (AECs) to dampen JAK-STAT signal transduction. Building upon this model, the current investigation sought to address gaps in knowledge surrounding EV uptake. We utilized a sensitive flow cytometry–based assay, confocal imaging, and pharmacologic and molecular inhibition to characterize and quantify AEC uptake of MVs in relation to that of macrophages known to be internalized by receptor-dependent and -independent endocytosis. We also identify relevant alterations within the alveolar microenvironment that can both positively and negatively regulate MV uptake. Our studies provide new
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insights into the mechanisms governing MV uptake and its modulation.

Results

Characteristics of AEC uptake of AM-derived MVs

We wished to model cell-cell communication between the two major and neighboring cell types at the alveolar surface by studying AEC uptake of AM-derived MVs. The relevant target cell of interest is the type I AEC, as it comprises 95% of the alveolar surface area (12), and rat L2 AECs were selected as a suitable cellular model that shares many of their phenotypic features (13, 14). Most experiments employed MVs isolated from the mouse MH-S AM cell line, although key parameters of uptake were verified using MVs isolated from primary rat AMs (Fig. S1). To measure uptake by AECs of AM-derived MVs and to study the mechanisms and modulation of uptake, we designed a quantitative flow cytometric assay as described under “Experimental procedures” and outlined in Fig. 1A. AECs took up AM-derived PKH-26–labeled MVs in a time-dependent (Fig. 1B) and concentration-dependent (Fig. 1C) manner. Uptake was very rapid (Fig. 1B). Additionally, uptake assessed by flow cytometry (Fig. 1C) and confocal microscopy (Fig. 1D) was temperature-dependent, indicating a metabolically active process. With sufficiently high concentrations of MVs, 100% of AECs demonstrated fluorescence shift by flow cytometry (example shown in Fig. 1A).

Attenuation of inflammatory signaling in AECs by AM-derived MVs depends on their internalization

We previously demonstrated that SOCS3 protein delivered in AM-derived MVs to AECs inhibited STAT3 phosphorylation and STAT3-dependent gene transcription both in vivo and in vitro (11, 15). LPS on the surface of Gram-negative bacteria is a critical initiator of inflammatory signaling implicated in acute lung injury (16) and recognized to activate STAT3 signaling (17). To confirm that MV-mediated inhibition of inflammatory signaling was dependent on active uptake of MVs, AECs were incubated with MVs at either 37 or 4 °C before the addition of LPS at 37 °C and subsequent determination of expression of the STAT3-dependent gene monocyte chemoattractant protein-1 (Mcp-1/Ccl2) (Fig. 1E). MV delivery at 37 °C resulted in inhibition of LPS-induced Mcp-1 expression, but this effect was lost when MVs were provided to AECs at 4 °C (Fig. 1E). Our standard uptake assay employs MVs whose membrane lipids are labeled with PKH-26. Because SOCS3 is a cytosolic protein, we sought to verify vesicular delivery of labeled cytosolic contents from source AMs by incubating AMs with carboxyfluorescein succinimidyl ester (CFSE) before isolation of MVs. AECs given CFSE-labeled MVs demonstrated increases in fluorescence (Fig. S2) similar to the increases in those given membrane-labeled MVs, as shown in Fig. 1. These data indicate that active internalization of AM-derived MVs is necessary and sufficient to provide this anti-inflammatory signal to AECs.

AECs internalize AM-derived MVs via dynamin-dependent endocytosis

To interrogate mechanisms of MV uptake in this model, AECs were treated with a panel of commonly used inhibitors reported to interfere with EV uptake in various cell types and experimental contexts (Fig. 2A). The greatest magnitude of inhibition was observed with the dynamin inhibitor dynasore, which is known to inhibit endocytosis (18). Other agents affecting a variety of intracellular processes known to be involved in endocytosis (the PI3K pathway, intracellular calcium, tyrosine kinases, and actin polymerization) had significant yet less marked effects on MV uptake. Inhibitors targeting lipid raft-mediated endocytosis (m-β-CD) by AECs and AEC recognition of MV-containing phosphatidylserine (annexin V) had little effect on MV uptake. We verified that these concentrations of m-β-CD depleted cellular cholesterol (using a commercially available colorimetric assay) and unlabeled annexin V competitively inhibited labeled annexin V binding to phosphatidylserine present on MVs (using flow cytometry) (data not shown). Because of the apparent critical importance of dynamin inferred from these inhibitor studies, we sought to further evaluate its role using a molecular approach. Indeed, knockdown of dynamin mRNA (Fig. S3) and protein (Fig. 2C) with siRNA resulted in significant inhibition of MV uptake (Fig. 2B), confirming that AM-derived MVs are internalized by dynamin-dependent endocytosis in AECs.

AECs internalize MVs independent of ligand-receptor interactions

Both the uptake of EVs (19–22) and the ensuing functional consequences thereof (23–25) have been reported to depend on interactions between EV ligands and cell surface receptors. Although annexin V showed no ability to inhibit MV uptake in our model (Fig. 2A), a role for AEC receptors other than those recognizing phosphatidylserine present on MVs was still possible. Because receptor-mediated endocytosis classically requires clathrin (26–28), we also tested the effects of clathrin heavy chain (Chc) knockdown. In contrast to our results with dynamin knockdown, siRNA targeting clathrin (Fig. 2C and Fig. S3) failed to inhibit uptake of MVs (Fig. 2B), suggesting the possibility of a receptor-independent mechanism. As an additional approach to determining whether MV uptake requires an initial cell surface-ligand interaction, we employed an approach that has been used previously to characterize classical uptake mechanisms for macromolecules and compared the uptake characteristics of MVs with those of albumin and dextran. AECs were incubated with MVs at 4 °C (which permits surface binding but not internalization) and then washed and subsequently placed at 37 °C in fresh medium to allow active internalization of any already bound MVs (Fig. 2D, middle condition). In parallel, we used identical conditions to examine the uptake of fluorescently labeled albumin (Fig. 2E) and dextran (Fig. 2F), known to be internalized in AECs by receptor-mediated and fluid-phase endocytosis (macropinocytosis), respectively (29–32). As expected of a macromolecule whose internalization depends on a surface receptor of finite abundance, albumin exhibited binding-dependent uptake (Fig. 2E), which was susceptible to competitive inhibition by excess unlabeled albumin at 37 °C (Fig. 2H). This was in contrast to uptake of fluorescently labeled dextran, which, as expected, showed no dependence on initial surface binding (Fig. 2F) and no competitive inhibition (Fig. 2I). In contrast to albumin, uptake of
labeled MVs showed no dependence on an initial binding event (Fig. 2D). These results also confirm that fluorescence increases in this model are not attributable to nonspecific dye transfer to AECs from labeled MVs. In addition, uptake of labeled MVs did not exhibit competitive inhibition by the addition of excess unlabeled MVs at 37 °C (Fig. 2G). Although AEC uptake of MVs was only moderately sensitive to the macropinocytosis inhibitor amiloride (Fig. 2A), uptake of dextran was markedly inhib-
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Edited by amiloride (Fig. 2J). In contrast, dextran uptake showed a degree of inhibition by dynasore (Fig. 2J) that was less than that of MVs (Fig. 2A). Taken together, these results demonstrate that AECs internalize AM-derived MVs via endocytosis independent of ligand-receptor interactions. In these respects, it is similar to macropinocytosis. However, the limited sensitivity of MV uptake (as compared with that of the classic fluid-phase endocytosis indicator dextran) to amiloride and the heightened sensitivity to dynasore together suggest internalization by processes distinct from those that are operative for macromolecules.
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Lung lavage fluid protein enhances AEC uptake of AM-derived MVs

Cells studied in tissue culture medium are lacking many unique components of their native microenvironment. To evaluate whether constituents of the normal alveolar environment influence AEC uptake of AM-derived MVs, lungs of naive rats were lavaged with cell culture medium, and previously isolated and labeled MVs were suspended in the resulting lung lavage (LL) fluid. We then compared uptake by AECs incubated with equal numbers of MVs resuspended in either LL or medium alone. AEC uptake of MVs was enhanced in the presence of LL compared with medium alone (Fig. 3, A and B). To gain insight into the nature of the alveolar constituents responsible for enhanced MV uptake, we tested the effects of LL subjected to either 56 °C heat inactivation (Fig. 3A) or 3-kDa size-exclusion centrifugal filtration (Fig. 3B). Components of LL responsible for enhancement of MV uptake were heat-sensitive and greater in size than 3 kDa. As for basal uptake, the enhancement promoted by either unfraccionated LL (Fig. 3A) or the >3-kDa fraction thereof was also dynamin-dependent and independent of surface binding (Fig. 3B). These results collectively indicate that protein(s) present on the luminal surface of the lung potentiate the dynamin-dependent fluid-phase endocytosis of MVs by AECs, rather than activating an additional uptake process.

Albumin in tissue fluids enhances fluid-phase uptake of MVs in AECs

As LL fluid contains a large number of proteins > 3 kDa, we tested the effects on MV uptake of a subset of cytokines and growth factors that have well-recognized immunomodulatory effects on AECs, including IL-1β, IL-6, IL-10, TNF-α, IFN-γ, and GM-CSF. In addition, we previously observed that the lipid mediator prostaglandin E2 (PGE2) enhanced AM packaging of SOCS3 into MVs (11), and we thus also tested the effects of PGE2 on MV uptake. Because mediators could influence uptake in a variety of ways, we tested the effects of each mediator on uptake using three different experimental designs: 1) co-incubation with MVs, 2) 30-min pretreatment before MV delivery, and 3) 16-h pretreatment before MV addition. None of these mediators had significant effects on MV uptake under any of these three treatment protocols (data in Fig. 3C depict 30-min pretreatment; others not shown). Next, to examine whether protein(s) responsible for enhanced MV uptake in AECs were unique to the alveolar compartment, we also assessed the modulatory effect of rat peritoneal lavage (PL) fluid and FBS. PL also potentiated MV uptake in AECs, and this effect was abrogated with 56 °C heat inactivation (data not shown). Even FBS potentiated MV uptake by AECs (data not shown), underscoring the conclusion that protein(s) responsible for MV uptake enhancement are not unique to the lung. To test whether individual high-abundance proteins common to these body fluids are sufficient to potentiate uptake, we examined the effects of BSA and IgG. MV co-incubation with IgG prevented MV uptake (Fig. 3D), indicating that IgG is not the protein responsible for the uptake enhancement observed in these body fluids. However, BSA enhanced AEC uptake of MVs in a dose-dependent manner (Fig. 3E). To ensure that this effect could not be explained by BSA dequenching of fluorescence, PKH-labeled MVs were resuspended in either 5% BSA or medium alone and analyzed by flow cytometry; no difference in fluorescence was detected (data not shown), excluding a dequenching action by BSA. In contrast to the effects of BSA co-incubation with MVs, AECs pretreated with BSA, washed, and then incubated with MVs did not demonstrate enhanced uptake (Fig. 3E). That uptake enhancement by BSA required its co-incubation with MVs suggests that this action is not attributable to preconditioning of the recipient cells. Rather, these results reveal that AEC uptake of MVs is potentiated by the concomitant presence of extracellular albumin.

Cigarette smoke extract inhibits AEC uptake of MVs independent of cytotoxicity

Cigarette smoke (CS) is well-recognized to elicit lung inflammation (33) and to injure AECs (34, 35). Our prior work showed that exposure of mice to CS in vivo alters AM packaging of SOCS3 into secreted MVs (11). We therefore considered the possibility that this clinically relevant environmental exposure may also modulate MV uptake. To test this, we incubated AECs for 2 h with cigarette smoke extract (CSE), a commonly used method to examine the effects of CS in vitro, before the addition of labeled MVs. Over a concentration range of 0.5–8%, CSE inhibited MV uptake in a dose-dependent manner, with complete inhibition occurring at 4 and 8% CSE (Fig. 4A). To assess AEC cytotoxicity from CSE at these concentrations and incubation times, a lactate dehydrogenase (LDH) release assay was performed. At no dose employed did 2-h CSE treatment of
AECs cause any significant release of LDH (Fig. 4B). Additionally, AECs treated for 2 h with CSE, washed, and then placed back in CSE-free serum-containing medium overnight completely regained their capacity to take up subsequently added MVs (data not shown). That the inhibition by CSE of MV uptake is reversible further argues against a role for cytotoxicity in this effect.

**Inhibition of MV uptake by CSE is independent of ATP depletion, LPS contamination, and proteasome inhibition**

CS is recognized to promote both acute and chronic pathophysiologic changes in the lung, which reflect the hundreds of its constituent organic and inorganic compounds capable of disrupting cell functions through a variety of actions (reviewed...
in Ref. 36). Thus, we evaluated a number of potential mechanisms by which CSE might inhibit MV uptake. Because MV uptake is energy-dependent, we measured ATP levels in AECs treated with CSE and compared levels with those obtained following treatment with oligomycin, an inhibitor of ATP synthase in the mitochondrial respiratory chain. As expected, a 2-h treatment with oligomycin at 10 μM reduced AEC ATP levels by ~50% (Fig. 4C) yet had minimal impact on MV uptake (Fig. 4D). By contrast, AEC treatment for 2 h with 4% CSE was sufficient to completely inhibit MV uptake, yet it increased ATP levels by 75%, the latter consistent with previous reports (37). From this we conclude that ATP depletion does not explain

**Figure 4. Cigarette smoke extract inhibits MV uptake in AECs.** A, AECs were incubated in CSE diluted in culture medium at the indicated concentrations for 2 h. CSE was removed, MVs resuspended in fresh medium were given to AECs for 1 h, and uptake was assessed with flow cytometry. Data are from at least two experiments normalized to MVs in medium alone (dashed line). *, p < 0.05 versus medium; #, p < 0.05 versus 1% CSE; & p < 0.05 versus 2% CSE (one-way ANOVA). B, LDH cytotoxicity assay of AECs treated with the indicated concentrations of CSE for 2 h. Data are from one experiment representative of two experiments normalized to positive control (Triton X-100). No significant differences were observed in LDH levels from CSE-treated cells versus medium (one-way ANOVA). C, ATP concentrations were determined in AECs treated with CSE or oligomycin for 2 h. Data are mean ± S.E. (error bars) from three experiments normalized to AECs in serum-free medium (dashed line). D, MV uptake in AECs assessed following treatment with CSE in parallel with oligomycin. E, AECs were treated with LPS at the indicated concentrations for 2 h before MV uptake determination. *, p < 0.05 versus medium (two-way ANOVA). Data are from one experiment (D) and one experiment representative of three experiments (E) normalized to gMFI of untreated AECs in medium (dashed line). F, AECs were preincubated with proteasome inhibitors MG 132 or bortezomib at the indicated concentrations for 2 h before subsequent wash and incubation with CSE for an additional 2 h. *, p < 0.05 versus 2% CSE (no inhibitor); all conditions significant (p < 0.05) versus MVs in medium. Data are from two experiments normalized to gMFI of AECs treated with MVs in medium (dashed line) (two-way ANOVA).
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CSE-mediated uptake inhibition. LPS is a potent pro-inflammatory stimulus known to contaminate CS (38) and to alter numerous epithelial cell functions (17, 39, 40). However, a 2-h treatment of AECs with LPS had no effect on subsequent uptake of labeled MVs (Fig. 4E). CSE has been reported to induce protein degradation through the ubiquitin-proteasome complex (41). Given the demonstration that proteins involved in endocytosis are targeted for degradation by environmental stimuli (42), we examined the effects of the proteasome inhibitors MG-132 and bortezomib on CSE-mediated uptake inhibition; however, neither restored vesicle uptake in CSE-treated AECs (Fig. 4F), suggesting that CSE-mediated uptake inhibition is not explained by proteosomal degradation of proteins facilitating endocytosis.

CSE-mediated oxidation of AECs inhibits MV uptake

CS also contains a myriad of oxidant moieties and is well-known to stress cells in part via oxidant-mediated mechanisms. To assess whether CSE-mediated inhibition of MV uptake involves oxidant stress, AECs were treated with the widely used antioxidant N-acetyl cysteine (NAC) before and during MV delivery (Fig. 5). At all doses of CSE, NAC completely prevented inhibition of MV uptake (Fig. 5A), demonstrating that CSE inhibits uptake of MVs through oxidation. This inhibitory effect, however, was not reproduced by AEC treatment with 1000 µM hydrogen peroxide (Fig. 5B), the maximal concentration at which it was non-toxic to AECs, suggesting that these inhibitory effects are not generalizable to all oxidant species, but rather are attributable to particular oxidants present in CS.

Inhibition by CSE of AEC uptake of MVs reflects oxidative disruption of actin polymerization

To investigate the potential oxidation targets of CSE, we first assessed its specificity for MV uptake disruption by examining the effects of CSE on uptake of labeled albumin and dextran (Fig. 5, C and D). CSE treatment also resulted in marked inhibition of albumin and dextran uptake, indicating that CSE targets cellular processes required for internalization of both macromolecules and MVs. One such candidate is actin assembly. MV uptake by AECs was disrupted with the actin polymerization inhibitor cytochalasin D (Fig. 2A), and actin assembly is recognized to participate in both receptor-mediated endocytosis and fluid-phase endocytosis (reviewed in Ref. 43). Moreover, interactions between the actin network and dynamin, implicated in MV uptake by data in Fig. 2 (A and B), facilitate various forms of endocytosis (44–47). Thus, we hypothesized that inhibition by CSE of MV uptake is attributable to oxidation-mediated disruption of actin polymerization. To test this, AECs were exposed to CSE with and without NAC before delivery of MVs, and polymerized actin was assessed relative to actin monomers by fluorimetry (Fig. 5E) and confocal microscopy (Fig. 5F and supporting files). AECs exposed to CSE exhibited a considerable reduction of actin polymerization with apparent preservation of actin monomers. As it did for MV uptake, NAC treatment also normalized polymerized actin relative to actin monomers in CSE-exposed AECs. Collectively, these data demonstrate that CSE-mediated inhibition of MV uptake occurs via oxidative disruption of actin polymerization essential for internalization.

Discussion

In an in vitro model of acute lung injury, we found that AM-derived MVs attenuated LPS-induced gene expression in AECs of an important pro-inflammatory chemokine and that this action required MV internalization. Utilizing this model, in this report we provide unique insight into endocytosis of EVs and its regulation. As MV internalization mechanisms remain incompletely characterized, we utilized quantitative flow cytometry, confocal imaging, and pharmacologic inhibitors as well as molecular knockdown to investigate the operative processes by which labeled MVs are internalized by AECs. In contrast to the prevailing understanding, based predominantly on studies of exosome uptake, we found that AEC uptake of MVs more closely resembles classic fluid-phase endocytosis than receptor-mediated endocytosis. Modulation of cell-cell communication by the extracellular environment is of fundamental importance in biology, but the dynamic regulation of EV uptake remains virtually unexplored. In this paper, we also demonstrate bidirectional modulation of MV uptake by the AECs comprising the lung’s alveolar surface in response to relevant environmental factors; AEC uptake was enhanced by extracellular albumin and inhibited by CSE, the latter via oxidation of actin networks. These findings reveal the complexity and importance of understanding tissue-specific dynamics when investigating cell-cell communication.

Knowledge of endocytosis mechanisms derives primarily from studies of macromolecule uptake, viral entry, and membrane protein trafficking, whereas understanding the role of such mechanisms in EV internalization is largely based on work with exosomes. EV uptake is generally considered to require an initial surface-binding event. However, to our knowledge, approaches to assess competitive inhibition and adsorption that are standard in endocytosis research have not been applied in a dedicated study of EV uptake. Reports implicating fluid-phase endocytosis as the predominant internalization mechanism for EVs (48–51) are in the minority. We found that uptake of AM-derived MVs by AECs was dependent on dynamin function and actin polymerization. Although MV uptake in our system shared with classic fluid-phase endocytosis of dextran a lack of competitive inhibition or dependence on ligand-receptor interactions, it was less sensitive to amiloride and more sensitive to dynasore. This indicates a process distinct from previously characterized EV uptake mechanisms that shares some, but not all, features of classic macropinocytosis. Further investigation into the molecular mechanisms that differentiate EV uptake from that of macromolecules is required.

Whereas receptor-independent uptake implies a degree of target cell non-selectivity that may appear biologically undesirable, it is pertinent to consider that AMs and AECs represent the lone cell types at the normal alveolar surface. This is in contrast to the predominantly receptor-dependent internalization observed for EVs isolated from serum or multicellular tissues (19–22, 24, 25). Receptor dependence of EV uptake, particularly that which is cell-specific, is an attractive feature from the perspective of targeted signaling or therapeutics. However,
it is logical to assume that the importance of such cell-specific EV targeting and internalization mechanisms depends on the proximity between source and target cell and cellular heterogeneity of the destined tissue. Our findings suggest the relevance of a form of EV transfer where cells in close proximity communicate without the need for surface receptor recognition.

The dynamics of the extracellular milieu is an important driver of the host response within any tissue, which is especially true in the lung, given its direct interface with the outside environment. Our previous publications demonstrated that soluble bioactive mediators relevant to alveolar homeostasis in acute lung injury can influence the consequences of EV-mediated information transfer by rapidly altering MV packaging of SOCS3 cargo by AMs (11, 15). Soluble mediators and other environmental perturbations have been reported to also enhance secretion of EVs (52–58). However, the effect of bioactive mediators on MV uptake by target cells has not previously been investigated. We report here that MV uptake by AECs was not influenced by a panel of relevant mediators. However, uptake was enhanced by increasing albumin concentrations. Enhancement of macromolecule macropinocytosis by extrinsic factors has been described in cells stimulated with various growth factors (48, 59, 60) and by engagement of fatty acid receptors (61). There are also previous reports of physio-

Figure 5. Oxidation of AECs by CSE inhibits MV uptake via disruption of actin networks. AECs were pretreated with NAC for 30 min and subsequently co-treated with MVs and CSE for 2 h. A, assessment of MV uptake in CSE-treated AECs with and without NAC by flow cytometry. Data are from three experiments normalized to fluorescence of MVs in medium (dashed line). *, p < 0.05 versus corresponding CSE concentrations without NAC; #, p < 0.05 versus medium-untreated; & < 0.05 versus 1% CSE without NAC; $, p < 0.05 versus 2% CSE without NAC (one-way ANOVA). B, AECs were pretreated with 1000 μM H2O2 or CSE in the presence or absence of NAC before MV incubation. Data are from two experiments normalized to gMFI of MVs in medium (dashed line). C and D, AECs were pretreated with CSE with or without NAC before incubation with 647-albumin (1 μg/ml) (C) or 488-dextran (30 μg/ml) (D), and uptake was quantified with flow cytometry. For C and D, *, p < 0.05 versus all other conditions. There was no significant difference between other conditions (one-way ANOVA). Data are from three experiments normalized to gMFI of AECs receiving labeled macromolecule in medium (dashed line). E and F, AECs were grown on opaque, flat-bottom 96-well plates (E) or fibronectin-coated chamber slides (F) and treated with CSE for 2 h with and without NAC pretreatment and co-treatment followed by incubation with MVs at 37 °C for 30 min. Cells were washed and underwent gradual fixation with 4% paraformaldehyde and short permeabilization with 0.1% Triton X-100 before incubation with Alexa Fluor-488 conjugated phalloidin and 594-conjugated deoxyribonuclease I. Images are representative of three experiments. Data are normalized to cells in medium alone from three experiments. *, p < 0.05 versus medium, NAC, and CSE (one-way ANOVA). Error bars, S.D.
logic enhancement of EV uptake. Vasopressin enhanced uptake of EVs through activation of cAMP in the renal collecting duct (62), low pH in the tumor microenvironment enhanced membrane fusion (10), and oncogenic transformation was associated with enhanced uptake (48). However, our findings are distinct from these reports, given our observation that albumin-enhanced uptake of EVs is independent of membrane fusion, surface-ligand interactions (Fig. 3B), or preconditioning (Fig. 3E). Thus, enhanced uptake need not be a delayed consequence of functional changes within the target cell; rather, our data demonstrate that acute changes within the extracellular space can dynamically and directly enhance uptake of EVs in real time. Vascular leak is a generalized and fundamental consequence of inflammation in all tissue types and is responsible for the pulmonary edema that compromises gas exchange in the injured lung. We speculate that enhanced MV uptake by AECs in the presence of alveolar albumin that has leaked from the inflamed vasculature serves to dampen inflammation and promote homeostatic resolution in settings of injury to the lungs and other organs.

In contrast to the enhancement of MV uptake by albumin, to our knowledge, our finding of CSE-mediated inhibition of MV uptake is the first description of the extracellular environment inhibiting uptake of EVs. Importantly, CSE also inhibited classic receptor-dependent uptake of albumin along with macromolecules, both in an oxidant-dependent manner. In different experimental contexts, actin polymerization has been reported to be indispensable for non-EV endocytosis, including receptor-mediated and fluid-phase endocytosis (reviewed in Ref. 43). Recent results indicate a critical role for dynamin and actin (63–65), facilitated by cortactin (44–47), in coordinating cytoskeletal remodeling, thus linking our findings of inhibition of dynamin-dependent endocytosis of MVs with CSE-mediated oxidation of actin polymerization (Fig. 5, E and F). The sensitivity of uptake mechanisms in a type 1 AEC line to CSE is particularly relevant, as these cells are highly susceptible to oxidant stress due to their comparatively low expression of endogenous antioxidants (66). Therefore, disruption of receptor-mediated and fluid-phase endocytosis would be expected to interfere with diverse homeostatic processes and thus represents a new mechanism for smoking-related lung pathobiology.

Despite the critical role of endocytosis in cell biology, the dynamic impact of the extracellular environment mediating inhibition of even these classic forms of endocytosis requires further investigation. Pathologic contexts (67, 68), including oxidative stress (69) and CS (70), have been linked with enhanced endocytosis of membrane proteins and extracellular macromolecules. In contrast, others show that oxidant stress inhibits endocytosis of macromolecules (71–73) and CS inhibits phagocytosis (74), efferocytosis (75), and macromolecule uptake (76). These observed divergent effects of oxidant stressors on classic uptake processes underscore the necessity of understanding the impact of specific microenvironmental changes on EV uptake.

Internalization of MVs occurred within minutes (Fig. 1B), permitting very brisk modulation of inflammatory events within recipient AECs (Fig. 1E). Such rapid uptake of EVs has rarely been described previously. This rapidity of uptake, along with its real time augmentation by albumin (Fig. 3E) and inhibition by CSE (Fig. 4A), point to a nimble means of intercellular cross-talk that is exquisitely sensitive to microenvironmental changes. Although our work is primarily focused on MVs, results with albumin and dextran demonstrate more generally that the fundamental process of endocytosis is subject to modulation by factors such as CS in the extracellular microenvironment. Understanding the effect of other relevant tissue-specific environmental perturbations on EV uptake and the extent to which their effects are selective for EVs as compared with macromolecules will be an important area of future research. This endeavor will require further insight to distinguish between mechanisms governing the uptake of EVs and macromolecules, of different EV subtypes, and of EV uptake among different cell types.

**Experimental procedures**

**Reagents and inhibitors**

Rat cytokines (IL-6, GM-CSF, IL-1β, IL-10, IFN-γ, and TNF-α) were purchased from PeproTech (Rocky Hill, NJ). PGE₂ (Cayman Chemical) was dissolved in DMSO under N₂ and stored at ~80 °C. BSA (Sigma) was dissolved in PBS and passed through a 0.22-μm filter. CSE was prepared as described previously (77). LPS, oligomycin, methyl-β-cyclodextran, α-cyclodextran, annexin V, dynasore, nystatin, genistein, and NAC were purchased from Sigma. Cytochalasin D was purchased from Millipore. BAPTA-AM was purchased from Biomol. 5-(N,N-Dimethyl)amiloride-HCl was purchased from Enzo. The PI3K inhibitor LY 29402 and proteasome inhibitors MG-132 and bortezomib were purchased from Cayman Chemical. Alexa Fluor 488–conjugated phalloidin, Alexa Fluor 594–conjugated deoxyribonuclease I, Alexa Fluor 488–conjugated dextran (488–dextran) (70 kDa), and Alexa Fluor 647–conjugated albumin (647-albumin) were purchased from Invitrogen.

**Rat tissue lavage**

Pathogen-free female Wistar rats (125–150 g) were purchased from Charles River. Rat tissue lavage fluid (lung and peritoneal) and primary rat AMs were isolated and cultured as described previously (11, 15). Remaining cell-free rat LL or PL fluid was aliquoted, stored at ~80 °C, and used in separate MV uptake experiments. LL fluid was fractionated using 3-kDa size exclusion centrifugation columns (Millipore) spun at 4000 × g for 1 h. Heat inactivation of protein constituents was performed at 56 °C for 30 min. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

**MV isolation and fluorescent labeling**

Primary rat AMs isolated as above, or mouse AM-derived MH-S cells obtained from ATCC were used as the source of AM MVs. For MH-S AMs, cells were grown on polystyrene flasks in RPMI 1640 culture medium (Gibco-Invitrogen) containing 10% FBS and penicillin/streptomycin. Once confluent, cells were placed in serum-free medium for 90 min, and conditioned medium (CM) was collected. For primary AMs, cells
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were isolated and plated on polystyrene flasks in RPMI overnight without serum before CM collection. For CFSE (Sigma) labeling, MH-S cells in suspension were incubated with 10 μM CFSE at room temperature for 10 min. Cells were then pelleted with centrifugation (250 x g for 5 min) at 4 °C and resuspended in medium containing 10% FBS to quench remaining extracellular CFSE. Cells were then washed twice in serum-free medium, subsequently plated in culture flasks, and incubated at 37 °C for 90 min, and CM was subsequently collected. For all experiments, cell debris and apoptotic bodies were removed from CM with serial centrifugation (500 x g and 2500 x g, respectively) at 4 °C. MVs were pelleted with ultracentrifugation (17,000 x g for 30 min) at 4 °C. MVs were characterized as described previously (11) with some modifications. MV membranes were fluorescently labeled with PKH-26 (Sigma) lipophilic dye according to the manufacturer’s instructions. After dye incubation, excess dye was quenched with an equal volume of 0.22 μm-filtered FBS, and aggregates were removed by centrifugation (2500 x g for 12 min) at 4 °C. The remaining supernatant was washed twice with serum-free medium between ultracentrifugation spins (17,000 x g for 30 min) at 4 °C. The resulting labeled MVs were suspended in F12-K culture medium and used for quantitative uptake assessment and confocal microscopy. MVs were characterized and quantified using a BD FACSCanto II or Fortessa with light scatter and fluorescence channels set at logarithmic gain. Events selected for MV counting exhibited a characteristic size range determined with calibration beads (0.5 and 1.0 μm; Invitrogen), demonstrated increased fluorescence over background of unstained MVs, and were detected as events distinct from machine noise (identified as detected events common to filtered medium and CM). The ratio of MV/AECs used for uptake experiments typically ranged from 100 to 1000:1. We verified that the pertinent characteristics and mechanisms of uptake described in this paper also held true for MVs isolated using the ultracentrifugation protocol used in our previous publication (11) (17,000 x g for 160 min) (data not shown).

Uptake quantification

The spontaneously immortalized rat AEC cell line L2 (CCL-149) (ATCC) with predominant type I cell-like characteristics (13, 14) was used between passages 10 and 40. Cells were grown on 96-well plates for 4 days at 37 °C with 5% CO2 to a density of 30–50% confluence in F12-K medium (Gibco-Invitrogen) with 10% FBS and penicillin/streptomycin. Cells were washed three times with 1% BSA, and fluorescence was subsequently visualized and created with ImageJ software (National Institutes of Health) with the WCIT plugin. Representative images shown are maximum intensity projections.

Actin polymerization assay

L2 cells were plated at 90% confluence on flat-bottom, opaque 96-well plates for 24 h in serum-containing medium. Cells were subsequently washed and placed in serum-free medium with or without CSE and/or NAC at the indicated concentrations and durations listed in the figure legends before MV incubation for 30 min. Subsequently, cells were washed, fixed, permeabilized, and stained for F and G actin. Cells were washed three times with 1% BSA, and fluorescence was subsequently determined with a Spectramax fluorimeter with excitation settings at 495 and 590 nm and emissions at 519 and 617 nm, respectively. Average autofluorescence was subtracted for each condition and measured emission. The ratio of 519/617 for individual wells was calculated for F/G actin determination.

RT-PCR and siRNA knockdown

Total RNA and protein from subconfluent AECs grown on 6-well plates was isolated as described previously (15, 78). Relative gene expression of Mcp-1 (Ccl2), dynamin 2 (Dyn2), or Chc was determined using the Δ cycle threshold method with β-actin as the reference gene, as described previously (15, 78). Western blotting for dynamin (rabbit mAb, 1:1000; Abcam)
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and clathrin (rabbit mAb, 1:1000; Cell Signaling) and subsequent densitometric analysis were as described previously (15, 78). The primer sequences are listed in Table S1. After seeding onto 96-well plates, L2 cells were transiently transfected for 24 h using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. Cells were transfected with 100 nm siRNA targeting either rat Dyn2, Clec (ON-TARGETplus SMARTPool, Dharmaco), or negative control siRNA (scrambled sequence) (UCCUCGCAAGUGUCAAGGUU). At 96 h, MV uptake analysis was performed, and message and protein knockdown was verified with RT-PCR and Western blot, respectively.

Quantitative assays

An LDH cytotoxicity assay (Roche Diagnostics), a cholesterol assay (Cell Biolabs), and the Cell-Glo viability assay (Promega) to determine relative ATP levels were performed according to the manufacturers’ instructions.

Statistical analysis

Data are expressed as mean ± S.D. unless otherwise specified. Experimental treatments were performed in triplicate wells in each experiment. S.D. for controls was obtained by calculating the \( \text{fold change} \) between the mean control value from replicate experiments and individual control values. Data were analyzed using the Prism version 7.0 statistical program from GraphPad software, using either ANOVA with Tukey’s test, as specified previously (15, 78). The primer sequences are listed in Table S1.

Author contributions—D. J. S designed and performed experiments, analyzed the data, and wrote the manuscript. D. J. S. and M. P. G conceived the study and edited the manuscript. L. R. P., J. A. S., and J. M. S. contributed to experimental design and data analysis. J. M. S. and S. H. W. helped with reagent preparation. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgment—We thank Dr. Peter Mancuso for supplying the CSE used in these experiments.

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