

Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation

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Background: Exosomes are nanovesicles involved in intercellular communication. Their roles in various diseases are often contextual, depending on the cell type producing them. Although few studies hint toward the proinflammatory role of bronchoalveolar lavage fluid-derived exosomes in asthmatic progression, the cell types in lungs associated with exosome-mediated crosstalk and their resultant effects remain unexplored.

Objective: It is well established that exosome-mediated cellular communication can influence disease phenotypes. This study explores exosome-mediated cellular crosstalk between structural and immune cells in asthma pathogenesis.

Methods: Exosomes were isolated and detected from bronchoalveolar lavage fluid of control and asthmatic mice and were quantified by using a bead-based assay. Involvement of epithelial cells and macrophages were established by using immunohistochemical techniques in lung tissue sections. The role of IL-13 in exosome production was ascertained by using various *in vitro* and *in vivo* techniques. Exosome secretion was blocked in *in vitro* and *in vivo* settings by using a chemical inhibitor, and the effects on various asthmatic features were studied.

Results: Using combinatorial *in vitro* and *in vivo* approaches, we found that exosome secretion and production of exosome-associated proteins are higher in lungs of asthmatic mice compared with that seen in sham mice. Asthma is marked by enhanced secretion of exosomes by epithelial cells, but not macrophages, under the influence of IL-13. These epithelial cell exosomes induce proliferation and chemotaxis of undifferentiated macrophages. On the other hand, GW4869, which inhibited exosome production, resulted in a reduced population of proliferating monocytes and alleviation of various asthmatic features.

Conclusion: Under the influence of IL-13, epithelial cell-derived exosomes can induce enhanced proliferation and chemotaxis of undifferentiated macrophages in the lungs during asthmatic

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Key words: Asthma, allergy, exosomes, epithelial cells, IL-13, monocytes

Inflammatory airway diseases, such as asthma and chronic obstructive pulmonary disease, are often described in terms of the inflammatory cells and cytokines that drive airway remodeling, which is a hallmark of such diseases. Epithelial cells constitute the layer of bronchi and alveoli facing the lumen, which comes into direct contact with the external environment. It is now increasingly realized that epithelial cells actively contribute to the establishment and progression of these disorders by orchestrating the inflammatory response to exogenous stimuli, such as allergens or cigarette smoke. Although the epithelial genes and proteins that govern this process have been the subject of active research, less emphasis has been placed on the mechanism of communication between epithelial and inflammatory cells.

Exosomes are nanoscale membrane-bound vesicles that are increasingly implicated as an important communication tool among various cell types, such as trophoblasts and macrophages,¹ mast cells and dendritic cells,² and dendritic cells and T cells. Cell types that are frequently implicated in exosome production are reticulocytes,³ dendritic cells,⁴ lymphocytes,⁵ macrophages,⁶ and platelets.⁷ Exosomes can exert their effects by delivering their contents, which could be proteins, mRNAs, or microRNAs, to recipient cells.⁸ Although the role of individual components within exosomes is still unknown, a functional role of exosomes is well established in the manifestation of various diseases, such as cancer,^{9,10} diabetes,¹¹ renal disorders,¹² and inflammation.¹³

Interaction between the exosome and immune system is complex, resulting either in its activation or suppression in a context-dependent manner. For example, dendritic cell-derived exosomes were found to activate naive T cells and also facilitate transfer of functional peptide-MHC complex between DCs.¹⁴ Similarly, exosomes from macrophages infected with intracellular pathogens were found to secrete exosomes that stimulated a proinflammatory response.¹⁵ In contrast, tumor-derived exosomes have been shown to suppress the immune system through various mechanisms, including induction of apoptosis in activated T cells in a Fas-FasL-dependent manner,^{16,17} downmodulation of NKG2D expression, suppression of natural killer and CD8⁺ T cells,¹⁸ or expansion of spleen myeloid suppressor cells, which can suppress natural killer cells and probably CD4⁺ and CD8⁺ T cells.^{19,20} Similarly, fibroblasts from synovial fluid of patients with rheumatoid arthritis produced TNF- α -containing exosomes that delayed activation-induced death of infiltrated T cells, further potentiating the disease.²¹ Important roles for exosomes are also emerging in several other pathologic infections, such as HIV,^{22,23} *Staphylococcus aureus*-induced atopic dermatitis,²⁴ *Mycobacterium tuberculosis*-induced tuberculosis,²⁵ and leishmaniasis,²⁶ to name a few. Many proteins that play important roles

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Abbreviations used

AAI: Allergic airway inflammation
 AHR: Airway hyperresponsiveness
 APC: Allophycocyanin
 BALF: Bronchoalveolar lavage fluid
 FITC: Fluorescein isothiocyanate
 Hsp-70: Heat shock protein 70
 OVA: Ovalbumin

in asthma and allergic inflammation have been shown to be associated with exosomes, such as heat shock protein 70 (Hsp-70),^{27,28} TGF- β ,²⁹ and enzymes for leukotriene biosynthesis.⁶ However, the functional role of exosomes in mediating cell-cell communication in the context of airway diseases is yet to be explored.

Here we sought to identify cells that could produce exosomes that might influence the neighboring immune cells, such as monocytes leading to asthma pathogenesis. Here, for the first time, we report that bronchial epithelial cells are the major source of exosomes in lungs of asthmatic patients. We also show that exosomes released from bronchial epithelial cells induce the proliferation and chemotaxis of monocytes without affecting their differentiation. Moreover, we observed that drugs that inhibit exosome production led to alleviation of asthmatic conditions.

METHODS**Cell lines**

The bronchial epithelial cell line BEAS-2B, the monocytic cell line THP-1, and primary murine macrophages (MHS cells) were procured from ATCC (Middlesex, United Kingdom). Primary monocytes were purified from buffy coats of cord blood by using CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), as per the manufacturer's instructions. See the [Methods](#) section in this article's Online Repository at www.jacionline.org for a detailed description.

Animals

Male BALB/c mice (8-10 weeks old) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for a week before the experiments. All animals were maintained as per guidelines and protocols approved by the institutional animal ethics committee.

Antibodies

CD63, Alix, Tsg-101, Rab-27b, Hsp-70, and gluceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Fluorescently labeled CD81 (allophycocyanin [APC]), CD63 (fluorescein isothiocyanate [FITC]), MHC class II (FITC), and CD9 (FITC) were purchased from BD Biosciences (San Jose, Calif). CD11b (APC) was purchased from Abcam (Cambridge, United Kingdom), whereas E-cadherin was from eBioscience (San Diego, Calif). Goat anti-rabbit Alexa Fluor 488 (Life Technologies, Carlsbad, Calif) against CD63 was used at a 1:200 dilution for immunocytochemistry.

Development of an ovalbumin-sensitized mouse model of asthma and treatment of mice with GW4869

Mice were sensitized and challenged as described previously.³⁰ Mice were divided into 3 groups, as indicated, and each group ($n = 6$) was named according to sensitization/challenge/treatment: sham/PBS/vehicle (normal controls),

which was called the SHAM group; ovalbumin (OVA)/OVA/vehicle (allergic controls, OVA, chicken egg OVA, Grade V; Sigma, St Louis, Mo), which was called the OVA group; and OVA/OVA/GW4869 (allergic mice treated with 1.25 mg/kg per dose of GW4869, Sigma), which was called the GW 1.25 group.

Exosome isolation

Exosomes were isolated by using a series of centrifugation and ultracentrifugation techniques, as described elsewhere.³¹ See the [Methods](#) section in this article's Online Repository for a detailed description.

Exosome quantitation

Exosomes derived from bronchoalveolar lavage fluid (BALF) or BEAS-2B cells were quantitated with a micro-BCA kit (Sigma).

Semiquantitative detection of exosomes by using a bead-based assay

For semiquantitative detection of exosomes, antibody-coated beads were used, as described previously.³² Five thousand single beads were acquired for analysis. Hsp-70 or CD63 antibodies (Santa Cruz Biotechnology) were used for coating beads. See the [Methods](#) section in this article's Online Repository for a detailed description.

Western blotting

Total protein was extracted and resolved onto a polyacrylamide gel, which was then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk and then probed for various exosome markers, such as CD63, TSG-101, ALIX, RAB-5b, and RAB-27a.

Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) in the form of airway resistance was estimated in anesthetized mice by using the FlexiVent system (Scireq, Montreal, Quebec, Canada), which uses a computer-controlled mouse ventilator and integrates with respiratory mechanics, as described previously.³⁰ Final results were expressed as airway resistance with increasing concentrations of methacholine.

Lung histology

Formalin-fixed, paraffin-embedded lung tissue sections were examined for airway inflammation, goblet cell metaplasia, and subepithelial fibrosis with hematoxylin and eosin, periodic acid-Schiff, and Masson trichrome staining, respectively, as described previously.³³ See the [Methods](#) section in this article's Online Repository for a detailed description of inflammation scoring.

Cytokine measurements (IL-4, IL-5, and IL-13) in lung homogenate and total cell counts

Lung homogenates were used for ELISA of IL-4, IL-5, and IL-10 (BD PharMingen, San Diego, Calif) and IL-13 (R&D Systems, Minneapolis, Minn), as per the manufacturer's protocol. Results were expressed in picograms and normalized by protein concentrations. Total cell counts were estimated as described previously.³⁰ Briefly, BALF extraction was performed with 1.5 mL of cold PBS, recovered BALF was centrifuged, cell pellets were washed 3 times, and resuspended cell suspensions were used for total cell counting by means of flow cytometry.

Electron microscopy

For electron microscopy, exosomes were processed as previously described.³² Briefly, the isolated exosomes were fixed in 2% paraformaldehyde, followed by image acquisition on a transmission electron microscope (Philips CM 10) operated at 70 kV.

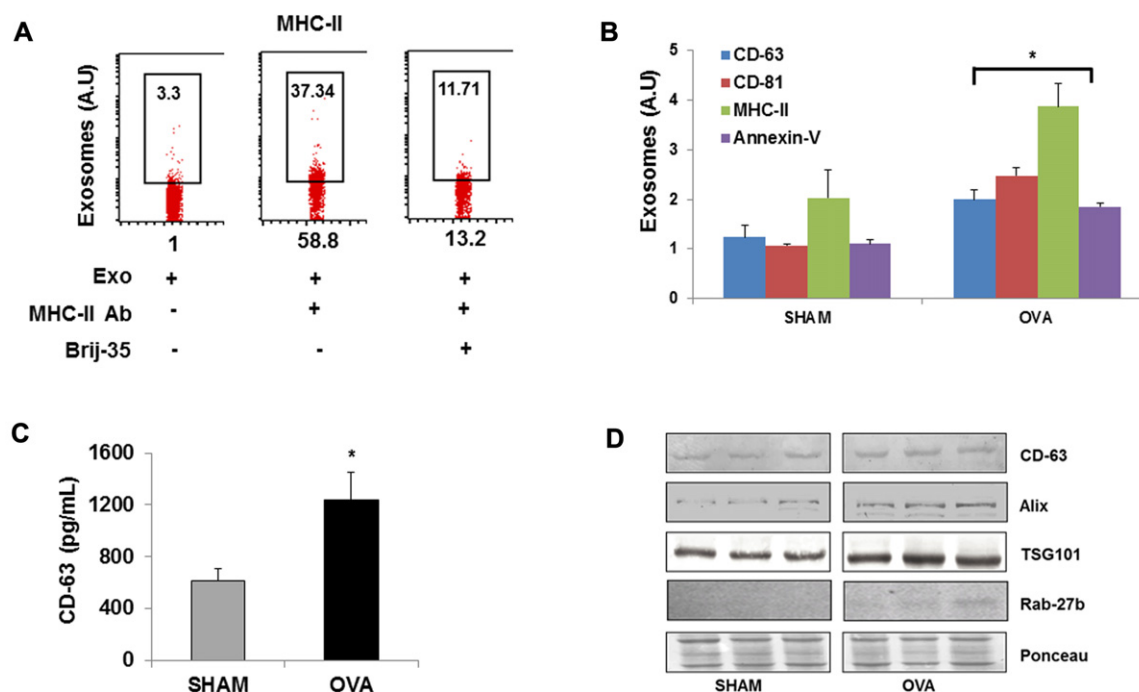


FIG 1. Increased secretion of exosomes in BALF and associated proteins in OVA-treated lung tissue. **A**, Beads incubated with BALF and Brij-35 showed diminished MHC class II levels. **Ab**, Antibody; **Exo**, exosomes. **B**, Secreted exosome-associated proteins were found to be higher in BALF from mice with AAI. **C**, ELISA for exosome-associated CD63 in BALF. **D**, Levels of CD63, Alix, Tsg-101, and Rab-27b in SHAM and OVA lung homogenates. Results are shown from 2 independent experiments. Fig 1, A-C, includes 5 mice in each group. Data are presented as means $\pm$ SEMs. * $P < .05$ versus the SHAM group. Fig 1, D, is representative of 2 independent experiments.

Immunohistochemistry

Paraffin-embedded tissue sections were used for preparation of 5- μ m tissue slides, and immunohistochemistry was performed, as described previously.³⁴ CD63 and Rab-5b antibodies were used at a dilution of 1:100.

Immunofluorescence

Paraffin-embedded tissue sections were used for preparation of 5- μ m tissue slides, and immunofluorescence was performed, as described previously.³³ CD63 (FITC), CD81 (phycoerythrin), and CD-11b (APC) antibodies were each used at a 1:100 dilution.

Transmigration assay

Overnight serum-starved THP-1 cells were incubated with 5 μ g of the indicated exosomes for 24 hours. The following day, cells were seeded in the top chamber of a Boyden chamber with or without monocyte chemoattractant protein-1 in the bottom chamber as a chemoattractant. Cells that migrated to the bottom chamber were counted after 3 hours of incubation under a hemocytometer and were verified by using flow cytometry.

Quantitation of microvesicle production by THP-1 cells

Exosome-treated THP-1 cells were seeded onto the top chamber of a Boyden chamber. Three hours later, the chamber was removed, and the secreted vesicles were counted in the media in the bottom chamber on a flow cytometer.

Statistical analysis

Data are expressed as means $\pm$ SEs. Significance of differences between groups was estimated by using the unpaired Student *t* test for 2 groups or

ANOVA with *post hoc* testing and Bonferroni correction for multiple group comparisons. Statistical significance was set at a *P* value of .05 or less.

RESULTS

Allergic inflamed lungs contain increased amounts of exosomes and associated proteins

To determine the status of exosome synthesis and secretion, we compared secreted exosomes in BALF from mice with or without allergic inflammation. For semiquantitative detection of exosomes, an equal volume of BALF from each group was incubated with anti-Hsp-70 antibody-coated beads (see Fig E1, A, in this article's Online Repository at www.jacionline.org), and total bound exosomes were detected with other surface proteins of exosomes. Coated beads were more efficient in detecting exosomes than native beads (see Fig E1, B1 and B2). Hsp-70, being a chaperone, could form complexes with multiple proteins. To ascertain the specific interactions of anti-Hsp-70 antibody with exosomes, we incubated antibody-coated beads with BALF in the presence of a mild detergent, Brij-35, which would solubilize the lipid portion of exosomes. Detergent treatment resulted in a significant reduction in the signal of exosomes, as detected by measuring bead-associated exosomal MHC class II levels (Fig 1, A). BALF exosome content was found to be higher in mice with allergic airway inflammation (AAI; Fig 1, B). ELISA for another well-established exosome-associated protein, CD63, in BALF confirmed the flow cytometric results (Fig 1, C). Pelletting down Hsp-70 beads incubated with BALF resulted in a significant reduction in CD63 levels, showing that CD63 is associated with

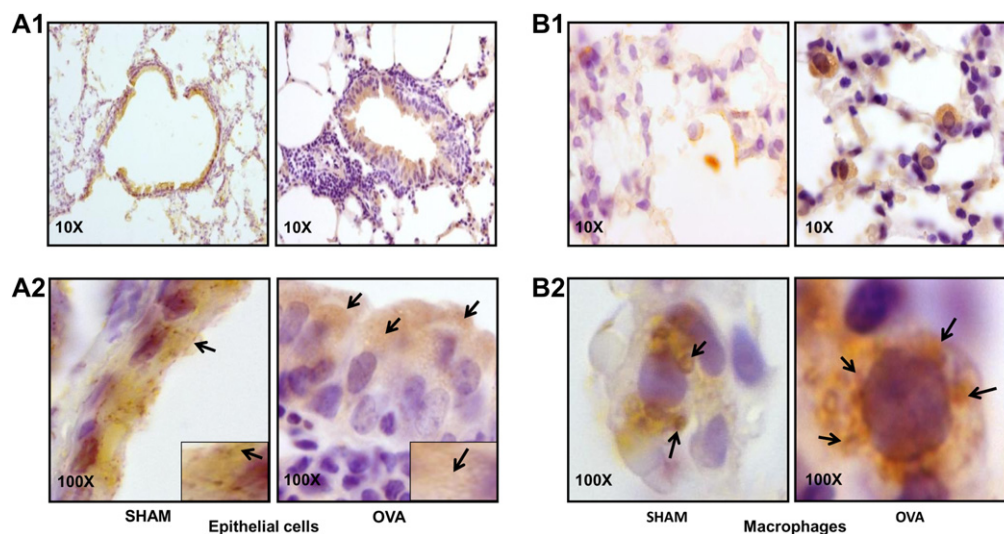


FIG 2. Exosome-associated proteins mostly localize to bronchial epithelial cells and macrophages. **A and B,** Immunohistochemistry for CD63 in tissue sections of SHAM and OVA mice indicating its presence in bronchial epithelial cells (A1 and A2) and macrophages (B1 and B2). Arrows indicate CD63⁺ compartments. Figures are representatives of 2 independent experiments.

Hsp-70 in a complex, which by definition is an exosome (see Fig E1, C). The specificity of the Hsp-70 antibody was detected by means of Western blotting, in which only Hsp-70 and no other protein was detected (see Fig E1, D). The expression of CD63, Rab-27b, Tsg-101, and Alix was compared between the total lung lysates from mice with or without AAI to determine whether the increased secretion of exosomes was associated with increased production of associated proteins in the lung. We found that exosome-associated protein levels were increased in the lungs of mice with AAI (Fig 1, D). BALF-derived exosomes were characterized for their size by using electron microscopy and dynamic light scattering (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org).

These results thus establish that there are increased levels of secreted BALF exosomes and exosome-associated proteins in the lungs of mice with AAI.

Exosome marker proteins mostly localize to bronchial cells and macrophages

A mouse model of AAI was developed, as described previously,³⁰ and confirmed by means of measurement of AHR and staining for infiltrated cells, mucus secretion, and collagen (see Fig E3, B and C, in this article's Online Repository at www.jacionline.org) to establish the involvement of lung cell types in exosome production. Immunohistochemical analysis was performed for some of the marker proteins of exosomes. CD63 and Rab-5b were found to be localized mostly to bronchial epithelial cells and macrophages (Fig 2 and see Fig E4, A and B, in this article's Online Repository at www.jacionline.org). Although bronchial epithelial cells are reliably defined by anatomic structure, double immunofluorescence with CD11b as a monocyte/macrophage and CD63 or CD81 as an exosome marker was performed (see Fig E4, C) to confirm localization to macrophages. Higher magnification micrographs of these CD63⁺ compartments in bronchial cells and macrophages showed a granular vesicle-like appearance in mice with AAI (see inset in Fig 2, A2 and B2),

suggesting increased exosome-secretory function by these cells during inflammatory conditions.

Because this was a novel observation, we looked at the levels of some of the well-reported exosome-associated markers in a related inflammatory airway disorder, chronic obstructive pulmonary disease, to determine whether this was a generalizable observation. Analysis of published microarray profiles of epithelial cells and alveolar macrophages (see Fig E5 in this article's Online Repository at www.jacionline.org) confirmed that the exosome-associated gene transcripts were higher in epithelial cells from smokers compared with those from healthy control subjects. However, this was not true for macrophages.

These results suggested that the bronchial epithelial cells and possibly macrophages in the lungs might be the key players involved with exosomal communication.

Effector T_H2 cytokines can increase the exosome secretion from epithelial cells *in vitro*

Because direct measurement of exosome secretion *in vivo* from individual cell types is not possible, we resorted to indirect cues by investigating the calcium content in lung epithelial cells and macrophages. Calcium content of a cell has been described to be one of the major factors regulating exosome secretion.³⁵ Epithelial cells from mice with AAI had significantly high ($P = .003$) calcium content, as measured by using Fluo-4AM dye, whereas macrophages did not exhibit much variation from SHAM mice (Fig 3, A and B), suggesting that epithelial cells might be the major cell type secreting exosomes during AAI. To confirm our *in vivo* findings and to establish an *in vitro* system mimicking asthmatic conditions, we treated BEAS-2B and MHS-1 cells with recombinant IL-13, an important T_H2 cytokine. IL-13 treatment significantly increased exosome secretion from epithelial cells over that seen with IL-4 and IFN- γ treatment (Fig 3, C). However, macrophages exhibited a marked reduction in exosome secretion by both IL-4 and IL-13 induction but not by the T_H1 cytokine IFN- γ (Fig 3, D). The validity of the system was tested by looking for the known effects of

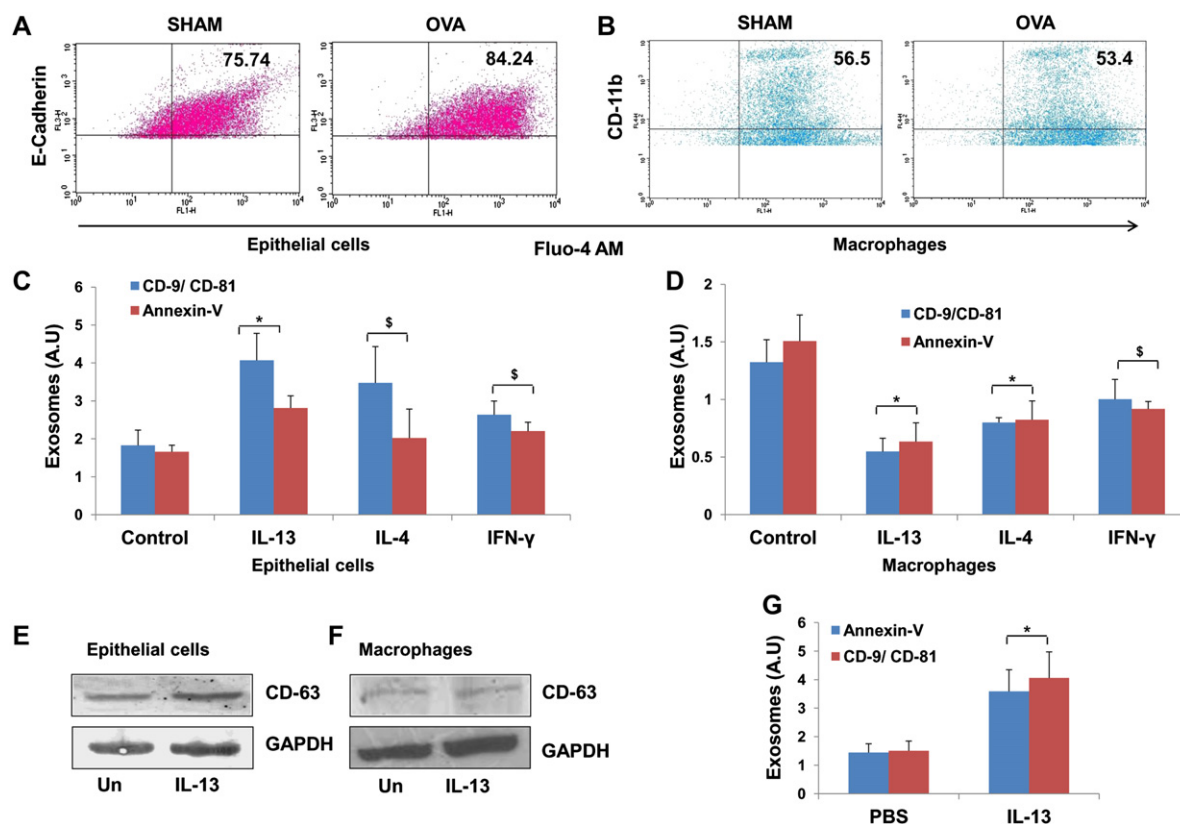


FIG 3. Opposing effects of IL-13 and IL-4 on exosome secretion from epithelial cells and macrophages. **A** and **B**, Intracellular calcium levels in the lung epithelial cells (Fig 3, **A**) and lung monocytes/macrophages (Fig 3, **B**). **C** and **D**, IL-13 or IL-4 increased exosome secretion from epithelial cells (Fig 3, **C**), whereas they decreased it from macrophages (Fig 3, **D**). **E** and **F**, CD63 levels in epithelial cells (Fig 3, **E**) or primary macrophages (Fig 3, **F**) on IL-13 treatment. **G**, Administration of IL-13 alone in mice increased exosome secretion in BALF. Data are represented as means $\pm$ SEs of 2 independent experiments with 4 mice in each group (Fig 3, **A** and **G**). * $P < .05$ and \$ $P > .05$ versus control, MHS cells, or PBS.

IL-13 and IL-4 on phagocytosis and nitric oxide production by macrophages (see Fig E6 in this article's Online Repository at www.jacionline.org), which were found to be negatively regulated by both of these cytokines, as described in the current literature. We also measured CD63 protein concentrations in IL-13-treated BEAS-2B cells, as well as in macrophages, and found that not only the secretion of exosomes but also the production of associated protein showed corresponding changes (Fig 3, **E** and **F**).

To validate the increase in exosome secretion by rIL-13, we intranasally administered rIL-13 to mice and then measured levels of the secreted exosomes in BALF. Our results showed that even in the absence of AAI, administration of rIL-13 alone was sufficient to significantly increase exosome production in BALF (Fig 3, **G**).

Exosomes from IL-13-induced epithelial cells, but not naive epithelial cells, induce monocyte proliferation

We established, through immunohistochemistry, calcium measurements, and *in vitro* experiments, that the major exosome-producing machinery during AAI was associated with bronchial epithelial cells in the lungs and that the environment is predominantly dominated by epithelial cell-derived exosomes.

Because we had already seen the negative effect of major T_H2 cytokines on the most common lung-resident immune cells (ie, macrophage function), we probed for the effect of these IL-13-treated epithelial cell-derived exosomes on monocyte/macrophage properties. Exosomes from BEAS-2B cells for transfer experiments were isolated by using sucrose density gradient centrifugation and were characterized by using immunofluorescence and immunoblotting (see Fig E7 in this article's Online Repository at www.jacionline.org).

For this purpose, the monocytic cell line THP-1 was treated with exosomes derived from IL-13-treated BEAS-2B cells, which showed a significant proliferation over THP-1 cells treated with exosomes from uninduced or LPS-treated BEAS-2B cells (Fig 4, **A**). To exclude the possibility of this being the result of residual IL-13, we independently treated THP-1 cells with rIL-13 and found that IL-13 was not able to drive the proliferation of THP-1 cells (see Fig E8, **A**, in this article's Online Repository at www.jacionline.org), thereby confirming that this effect was exclusive to exosomes. A probe for induction of differentiation by these exosomes by using various macrophage maturation markers, such as F4/80, CD11b, and CD14, revealed that although exosomes from LPS-treated BEAS-2B cells resulted in increased surface expression of these macrophage markers, the exosomes from IL-13-treated BEAS-2B cells kept them in an

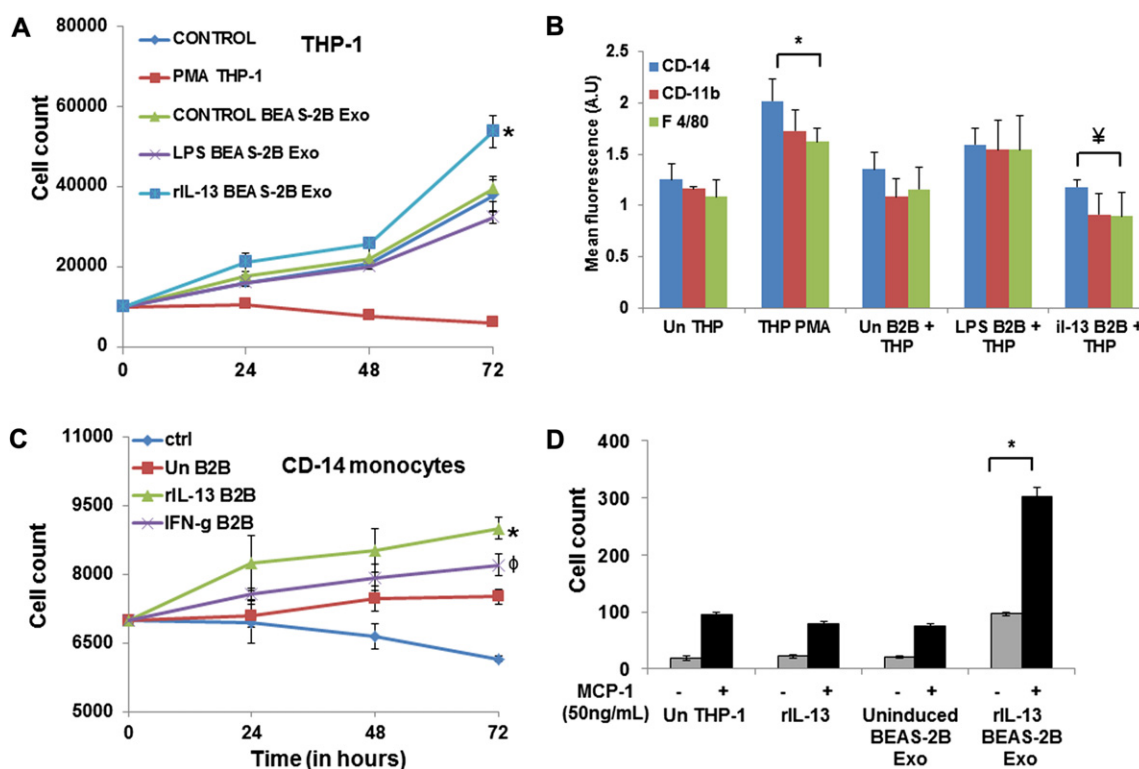


FIG 4. Exosomes from IL-13-treated BEAS-2B cells induce proliferation of THP-1 cells and primary monocytes but not their differentiation. **A** and **B**, Effect of exosomes collected from BEAS-2B cells with different stimulations on the THP-1 cell count (Fig 4, A) and primary monocytes (Fig 4, B). **C**, Levels of various macrophage markers on treatment with the indicated exosomes. **D**, Transmigration of THP-1 cells in response to monocyte chemoattractant protein 1 (MCP-1) on treatment with IL-13 and the indicated exosomes. Figures are plotted as means $\pm$ SEs of 2 independent experiments. * $P < .05$ compared with control THP-1 cells or monocytes. $\nless P < .05$ compared with phorbol 12-myristate 13-acetate THP-1 cells. $\phi P < .05$ compared with IL-13 BEAS-2B exosomes.

undifferentiated state while enhancing their rate of proliferation (Fig 4, C, and see Fig E9 in this article's Online Repository at www.jacionline.org). Because THP-1 is a readily proliferating cell line, to further validate these findings, we performed these experiments with cord blood-derived CD14⁺ monocytes, and similar results were observed. Exosomes from IL-13-treated BEAS-2B cells were found to induce proliferation in CD14 monocytes, even in the presence of macrophage colony-stimulating factor, which induced differentiation in control cells (Fig 4, B, and see Fig E10 in this article's Online Repository at www.jacionline.org). Exosomes from IL-13-treated BEAS-2B cells significantly increased the surface expression of monocyte marker CD11b while reducing the expression of CD64 on exosome-treated cord blood-derived monocytes, though no effect was observed on CD14 (see Fig E11 in this article's Online Repository at www.jacionline.org). Although no effect of IFN- γ -treated BEAS-2B cell-derived exosomes was observed on THP-1 cells (see Fig E8, B and C), significant proliferation was observed in CD14 monocytes, albeit lower than that seen in exosomes from IL-13-treated BEAS-2B exosomes.

Treatment of THP-1 cells with IL-13-induced BEAS-2B cell-derived exosomes enhanced their chemotactic potential, as measured by their enhanced transmigration to the bottom chamber of a Boyden chamber in response to monocyte chemoattractant protein 1 as a chemoattractant (Fig 4, D). Also, these IL-13-treated epithelially derived exosomes induced large release of

microvesicles from THP-1 cells, whereas exosomes from uninduced epithelial cells did not exhibit any such phenomenon and neither did IL-13-treated THP-1 cells (see Fig E12 in this article's Online Repository at www.jacionline.org). Thus epithelial cells under the influence of effector T_H2 cytokines can produce exosomes that modulate the phenotype of monocytes and probably enhance the inflammatory response.

Reduction in exosome secretion results in diminished population of proliferating monocytes in inflamed lung

We found that in *in vitro* settings, exosomes from IL-13-treated epithelial cells could induce monocyte proliferation. The proliferation of inflammatory monocytes is a prominent feature of the inflamed asthmatic lung. To test whether inhibiting exosome secretion *in vivo* could halt this proliferation, thereby exerting a protective effect, we used GW4869, a known inhibitor of exosome secretion.³⁶⁻³⁸ GW4869 effectively reduced the exosome secretion in BALF from mice with AAI in *in vivo* settings and from epithelial cells in a dose-dependent manner in *in vitro* settings (Fig 5, A, and see Fig E13, A, in this article's Online Repository at www.jacionline.org). GW4869 administration at a concentration of 1.25 mg/kg body weight before challenge in mice with AAI significantly reduced the monocyte/macrophage population (Fig 5,

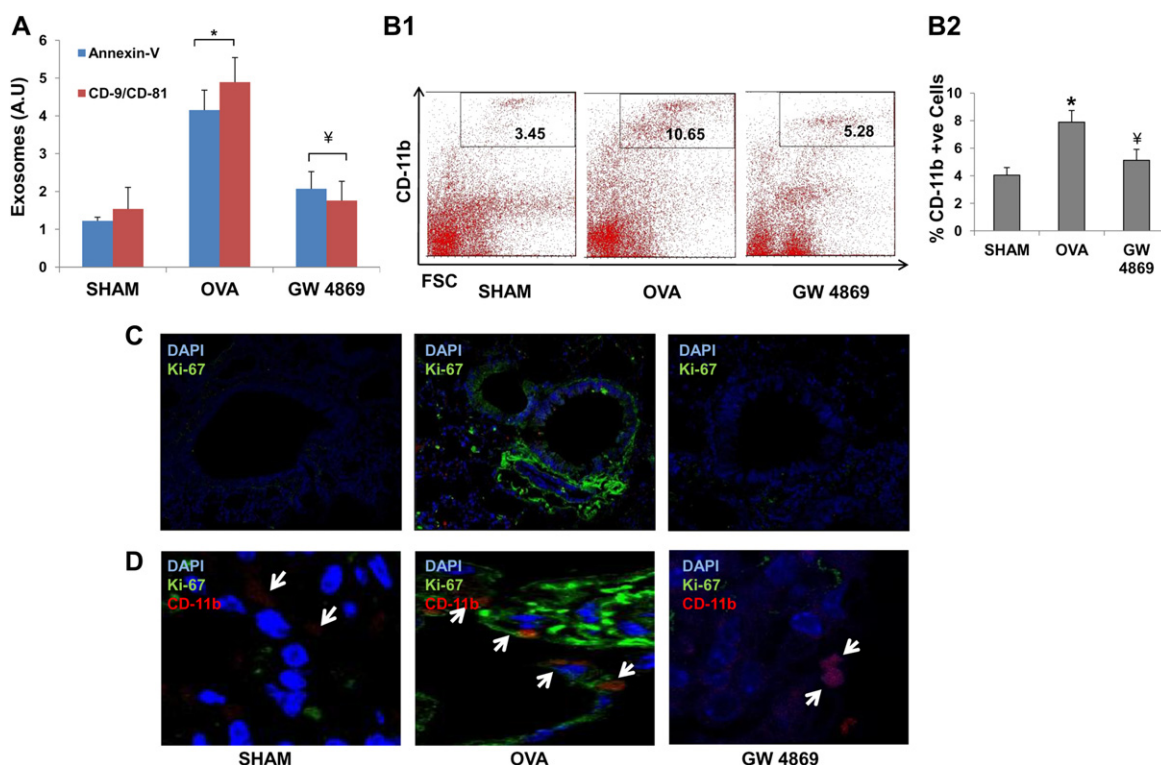


FIG 5. GW4869-mediated exosome reduction decreases monocyte/macrophage numbers in the lungs. **A**, GW4869 reduced exosome secretion *in vivo*. **B1**, CD11b⁺ monocyte/macrophage counts from lungs of SHAM, OVA, and GW4869-treated mice. **B2**, Pooled data from individual groups of Fig 5, **B1**. **C**, Proliferating Ki-67⁺ cells were localized in the lung. **D**, Proliferating monocytes were identified by using cells positive for both Ki-67 and CD11b in the lung sections. Fig 5, **C** and **D**, Blue = 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI), green = Ki-67, and red = CD11b. Data are represented as means $\pm$ SEMs of 2 independent experiments. * $P < .05$ versus the SHAM group. ¥ $P < .05$ versus the OVA group.

B1 and **B2**) in the lung. The proliferating cells were identified by performing immunohistochemistry for proliferation marker Ki-67 to confirm our hypothesis of monocyte proliferation. Most of the Ki-67⁺ cells were located near the vessels in OVA-challenged mice (Fig 5, **C**), mostly representing the infiltrated cells from the blood. To locate the proliferating monocytes, we simultaneously used CD11b and Ki-67 and found that there was a significantly high population of Ki-67⁺ monocytes in mice with AAI, which went down markedly after GW4869 treatment (Fig 5, **D**).

Reduction of exosome secretion by GW4869 can alleviate asthmatic features in a mouse model of bronchial asthma

Because our earlier results suggested that exosomes might be promoting inflammation in the lungs and that the exosome-reducing compound GW4869 decreases proliferating monocytes, we thought of testing its effect on other features of bronchial asthma.

We found that GW4869 administration significantly reduced methacholine-induced AHR (Fig 6, **A**) and other histopathologic features, such as inflammation (Fig 6, **B**, and see Fig E13, **C**) and mucus production (Fig 6, **C**). There was also a marked decrease in the number of infiltrated cells in BALF and levels of serum IgE (Fig 6, **D**) and various inflammatory cytokines, such as IL-13, IL-4, and IL-5 (Fig 6, **E**, and see Fig E13, **B**).

These results suggest that an inhibitor or inhibitors of exosome production have beneficial effects in experimental asthma. A summary of results has been illustrated in Fig 7.

DISCUSSION

There is increasing evidence that lung epithelial cells actively modulate the local immune response.^{39,40} Although secreted cytokines, such as thymic stromal lymphopoietin, IL-33, and IL-25,⁴¹⁻⁴³ have been shown to play critical roles, the potential role of exosomes has not been sufficiently investigated. This is a major lacuna in the field because exosomes have recently been described to importantly modulate the immune system in patients with other pathologic conditions.³⁹

Here we show that secretion of exosomes is increased during allergic inflammation, which might mediate increased intercellular signaling. Importantly, IL-13-treated epithelial cells secrete exosomes that stimulate inflammatory cell proliferation, and the drug or drugs that inhibit exosome production can dampen inflammation. Yet it is likely that exosomes play an important role in the maintenance of tissue health because they are detectable in appreciable amounts in BALF from control mice. In support, Bolasco et al⁴⁴ have shown that the double-knockout mice lacking the exosome biogenesis proteins Rab-27a and Rab-27b have an abnormal lung structure. We also found that the exosome inhibitor GW4869 promotes inflammation in OVA mice at higher doses (2.5 mg/kg body weight, data

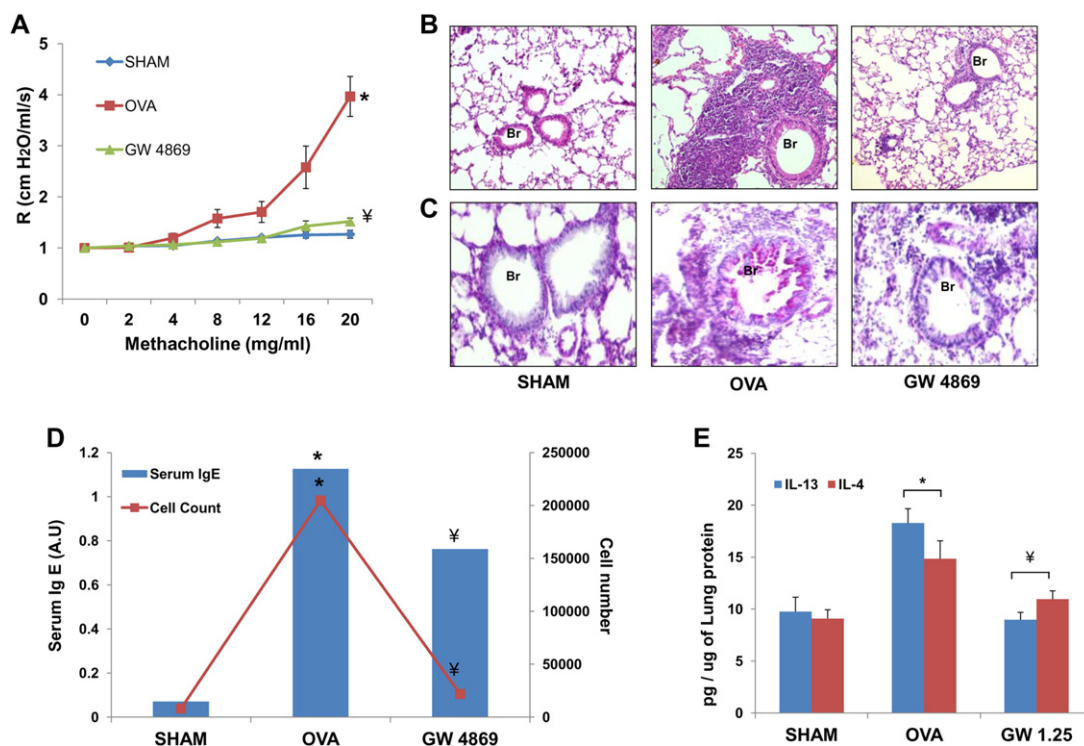
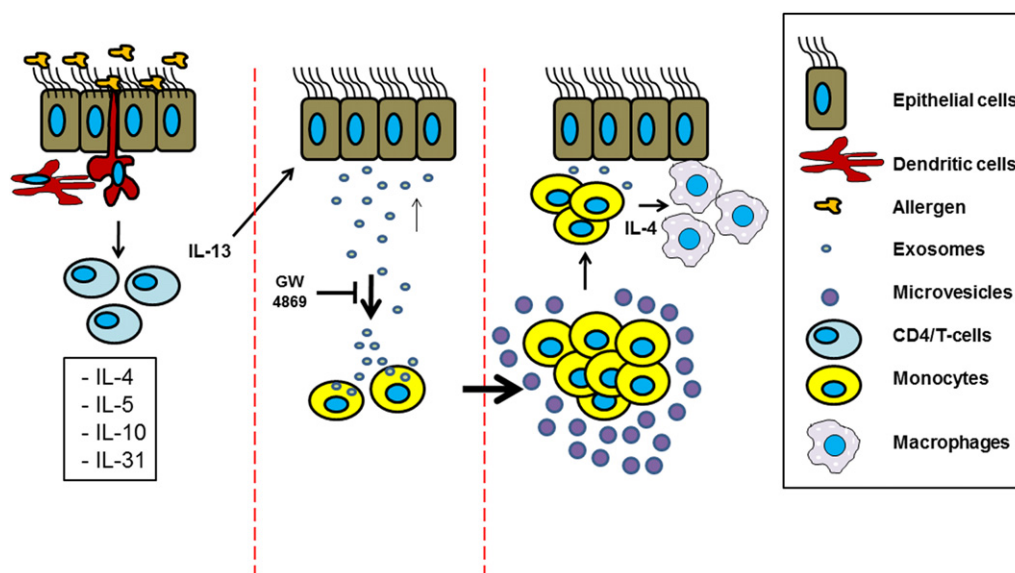


FIG 6. Reduction in exosome secretion in mouse lungs also alleviates asthmatic features. **A**, Airway resistance (R) with increasing concentrations of methacholine. **B** and **C**, Lung sections depicting the extent of airway inflammation (hematoxylin and eosin; Fig 6, **B**) and mucus metaplasia (periodic acid-Schiff; Fig 6, **C**). Stains are shown at $\times 20$ magnification. *Br*, Bronchus. **D**, Total cell count in BALF and serum IgE levels. **E**, IL-13 and IL-4 levels measured in total lung. Fig 6, **A**, **B**, and **E**, are plotted as means $\pm$ SEs of 2 experiments with 4 to 6 mice in each group. * $P < .05$ versus the SHAM group. ‡ $P < .05$ versus the OVA group.



not shown), suggesting that reducing the exosome level beyond a threshold could be fatal for the maintenance of tissue homeostasis.

The major lung cell types that expressed exosomal genes and proteins, as determined by using expression profiling and immunohistochemistry, were bronchial epithelial cells and

macrophages. Vesicular exosome-like structures were also visible in these cells. Epithelial cells have been shown to produce exosomes that display autoantigen to immune cells in another system as well, although in a different context.⁴⁵ Bronchial epithelial cells are at the interface of the environment and the lung tissue and might use exosomal communication to signal important changes in the environment to deeper tissue cells. Macrophages and dendritic cells, being the resident antigen-presenting cells, are important in mounting response and amplifying the signals that they received from epithelial cells. Calcium measurements suggested that there is activation of bronchial epithelial cells, but not macrophages, in the setting of allergic inflammation. In culture increased exosome secretion from alveolar macrophages treated with IFN- γ , but not with IL-13 or IL-4, suggests that lung macrophages might be important sources of exosomes in T_H1 but not T_H2 types of inflammation. However, this requires further confirmation.

Among the multitude of factors that could be responsible for the enhanced exosome secretion seen in the lungs of asthmatic patients, calcium seems to be an important factor because many studies have suggested that increased intracellular calcium plays an important role in exosome generation.^{35,46} We also found that increased exosome secretion from lung epithelial cells was associated with high intracellular calcium. We have previously shown that experimental asthma is associated with an increased hypoxic response,⁴⁷ which can in turn lead to high ceramide generation through activation of sphingomyelinase, thereby increasing exosome secretion. Accordingly, inhibition of exosome secretion by GW4869 was found to alleviate asthmatic features in our studies (Figs 6 and 7). However, additional effects of GW4869 beyond inhibition of exosome secretion cannot be excluded, although neutral sphingomyelinases are not known effectors of allergic inflammation.

For the first time, we show that bronchial epithelial cells orchestrate inflammation in asthmatic patients not only through increased exosome secretion but also through altered characteristics of exosomes, allowing them to induce local immune cell proliferation. Exosomes derived from IL-13-treated epithelial cells were able to induce proliferation of monocytes when compared with an equal amount of exosomes from control epithelial cells. Similar results were obtained with cord blood-derived primary monocytes. However, primary monocytes showed proliferation in response to exosomes from IFN- γ -treated epithelial cells as well, although to a significantly lesser extent. These were definitely exosome-mediated effects and not attributable to cytokine contamination because direct treatment of THP-1 cells with IL-13 did not induce their proliferation, although CD64 levels significantly decreased with exosomes from IFN- γ -treated epithelial cells, whereas direct treatment of IFN- γ increases its expression on monocytes. These observations have far-reaching implications because an epithelial cell-induced, exosome-mediated, local proliferation of progenitor immune cell populations (monocytes in this case), which could then differentiate along any of the many possible fates (eg, macrophage type M1 or M2 or dendritic cells), depending on the prevailing environment, is a very efficient system for responding to immune challenge (an innocuous foreign protein in this case). Local proliferation, rather than recruitment, might also be preferred as a first response because it would minimally affect systemic immune readiness. Our hypothesis is supported by the current literature, wherein many groups have reported the increase in local populations of

macrophages and dendritic cells, which essentially are derived from a myeloid lineage.^{48,49} We also found that in addition to inducing local proliferation, exosomes from IL-13-treated epithelial cells induce chemotaxis of monocytes, whereas neither uninduced epithelial cells nor rIL-13 had this effect.

Our data strongly support a model wherein cytokines, such as IL-13, induce a change in exosomal composition and thereby modulate inflammatory response. This could be a common motif across many clinically relevant situations and merits investigation. For example, it has recently been shown that exosomes isolated from mycobacteria-infected mice or cultured macrophages can recruit and activate immune cells in lungs.⁵⁰ It is reported that exosomes can carry chemokines encapsulated within them.⁵¹ It would be interesting to see whether exosome treatment can enhance the surface expression of chemokine receptors in monocytes and whether their effect is cell lineage specific.

In conclusion, our findings thus establish, for the first time, that effector T_H2 cytokines modulate intercellular signaling by altering the content and quantity of exosomes secreted by bronchial epithelial cells. In the case of asthma, IL-13-induced exosomal signaling might potentiate inflammation by inducing monocyte proliferation. This extends previous studies wherein leukotriene-synthesizing enzymes were found to be present in exosomes.⁶ Cutting off these communication lines by either inhibiting exosome secretion, as shown by us, or by hijacking these communications with artificial exosomes could be useful therapeutic strategies in patients with asthma and other inflammatory diseases.

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Key messages

- Epithelial cells are major exosome producers in the lung.
- IL-13 enhances exosome production and secretion from epithelial cells, which induce proliferation and chemotaxis of undifferentiated macrophages.
- Suppressing exosome secretion in the lung alleviates asthmatic features.

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METHODS

Cell lines

BEAS-2B cells were maintained in BEGM medium, whereas THP-1 and MHS cells were cultured in RPMI supplemented with 10% exosome-depleted FCS, streptomycin (50 µg/mL), and penicillin (50 IU/mL) at 37°C in a 5% CO₂ atmosphere. For culturing MHS cells, β-mercaptoethanol and glutamine were added separately to RPMI.

The purity of magnetic cell sorting-separated primary monocytes was greater than 95%, as assessed by means of flow cytometry with CD14-specific antibody (data not shown).

Exosome isolation

Exosomes were isolated by using a series of centrifugation and ultracentrifugation techniques, as described elsewhere,^{E1} with the modification wherein the supernatant from the 10,000g fraction was filtered with a 0.2-µm membrane before subjecting it to ultracentrifugation at 100,000g for 2 hours. The pellet was then washed with a large amount of PBS and then resuspended again in 200 µL of PBS, which was further purified by a sucrose density gradient centrifugation. For adaptive transfer experiments, the exosome pellet was resuspended in sterile PBS, whereas Laemmli buffer was used for Western blotting.

Semiquantitative detection of exosomes by using a bead-based assay

For semiquantitative detection of exosomes, antibody-coated beads were used, as described by Hong et al.^{E2} Briefly, 20,000 beads were incubated with a

10,000g supernatant of BALF or culture supernatant after washing in 2% BSA overnight. The next day, the bead-bound exosomes were detected by using surface proteins for exosomes or phosphatidylserine on their surface by means of flow cytometry.

Lung histology

The inflammation score was calculated by 2 independent observers who were blinded to the experiment, as described earlier, with few modifications.^{E3} Briefly, grades of 0 to 4 were given for no inflammation (0), occasional cuffing with inflammatory cells (1), when most bronchi or vessels were surrounded by a thin layer (1-2 cells deep; 2), and a moderate layer (3-5 cells deep; 3) or a thick layer (>5 cells deep; 4) of inflammatory cells. An increment of 0.5 was given if the inflammation fell between 2 grades, and the total inflammation score was calculated by addition of both the peribronchial and perivascular inflammation scores.

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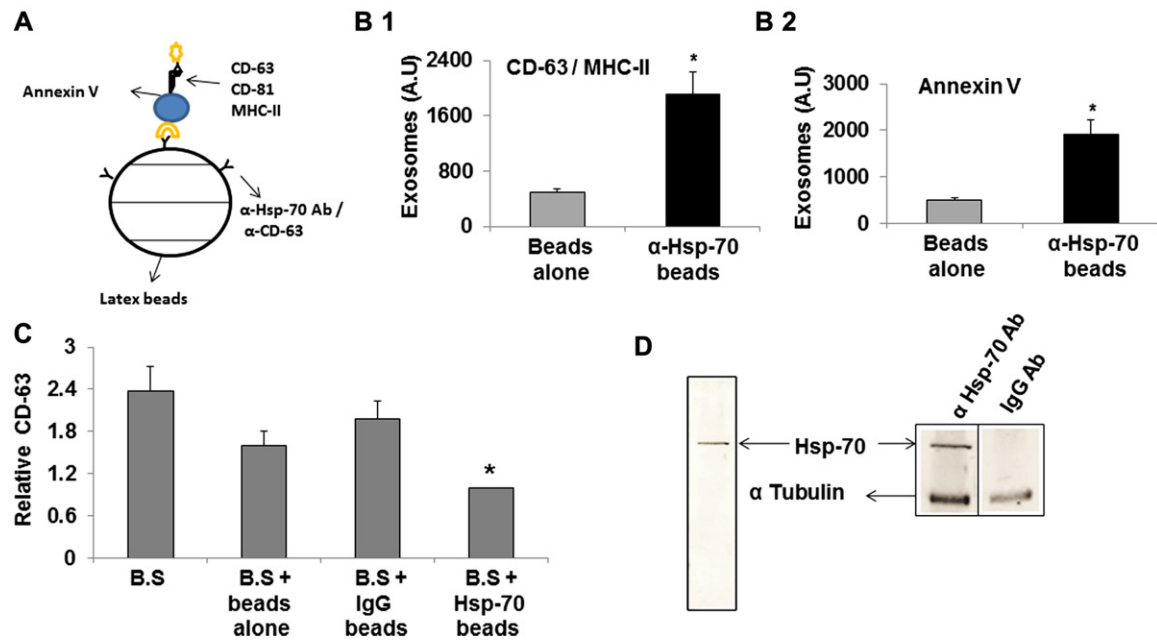


FIG E1. Isolation, characterization, and quantitation of exosomes from BALF. **A**, Scheme for semiquantitative detection of exosomes by using flow cytometry. **B**, Anti-Hsp-70 antibody-coated or uncoated beads were incubated with 200 μ L of BALF from each group, and the bound exosomes were detected with anti-CD63 and anti-MHC class II antibody (*B1*) or Annexin-V (*B2*). **C**, ELISA for CD63 in BALF after pelleting down beads. *B.S.*, BALF supernatant. **D**, Characterization of the Hsp-70 antibody used for coating latex beads.

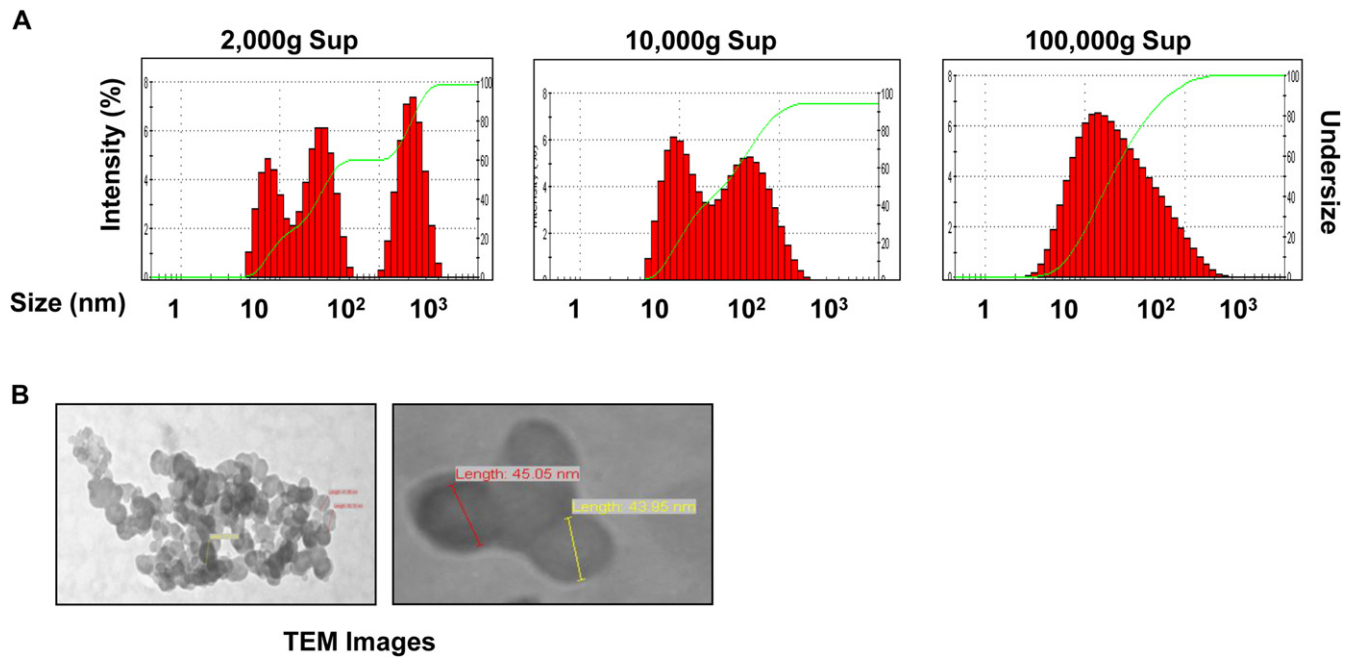


FIG E2. **A**, Dynamic light-scattering measurement for determining the particle size in the different fractions obtained during isolation of exosomes. **B**, Transmission electron microscopic (*TEM*) images for the isolated exosomes. Fig E2, *A*, is representative of 2 independent experiments.

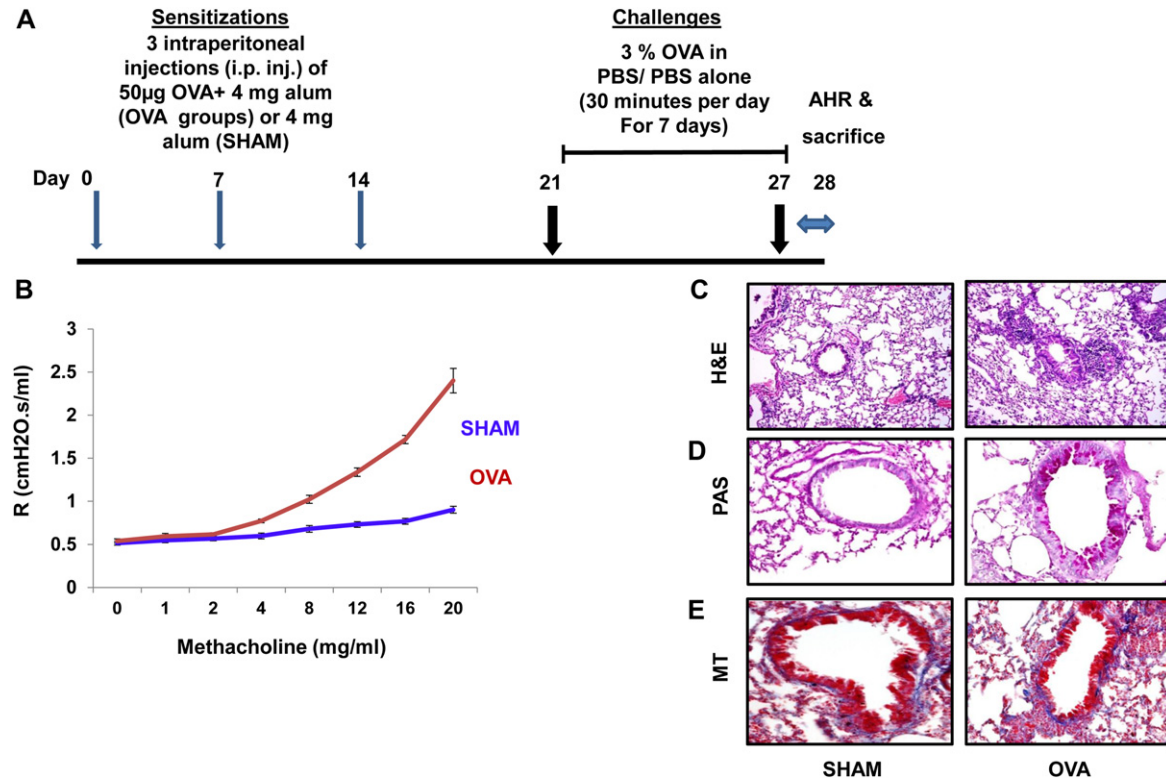


FIG E3. Development of the asthma model. **A**, Schematic diagram showing the protocol followed for developing the murine model of acute allergic asthma. **B-E**, Confirmation of model development by measurement of AHR (Fig E3, *B*), hematoxylin and eosin staining (Fig E3, *C*), periodic acid-Schiff staining (Fig E3, *D*), and Masson trichrome (MT) staining (Fig E3, *E*). Fig E3, *C* is shown at $\times 4$ magnification, and *D* and *E* are shown at $\times 20$ magnification. Fig E3, *B*, is plotted as the mean $\pm$ SE of 2 independent experiments. * $P < .05$.

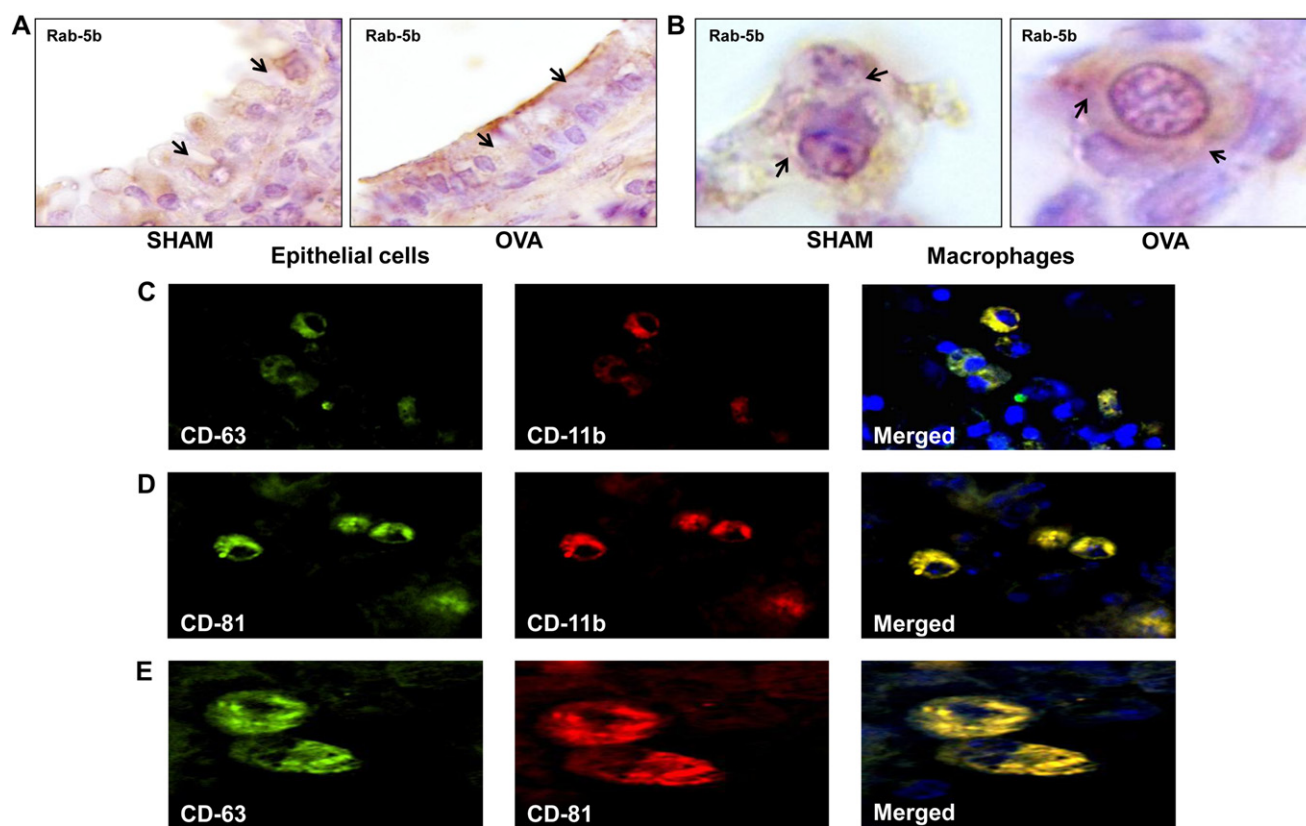


FIG E4. **A** and **B**, Immunohistochemistry for the exosome-associated protein Rab-5b in bronchial epithelial cells (Fig E4, **A**) and macrophages (Fig E4, **B**). **C-E**, Colocalization of macrophage markers CD11b and CD63 (Fig E4, **C**), macrophage markers CD11b and CD81 (Fig E4, **D**), and exosome markers CD63 and CD81 (Fig E4, **E**). Fig E4, **C** and **D**, are shown at $\times 60$ magnification and Fig E4, **A**, **B**, and **E**, are shown at $\times 100$ magnification.

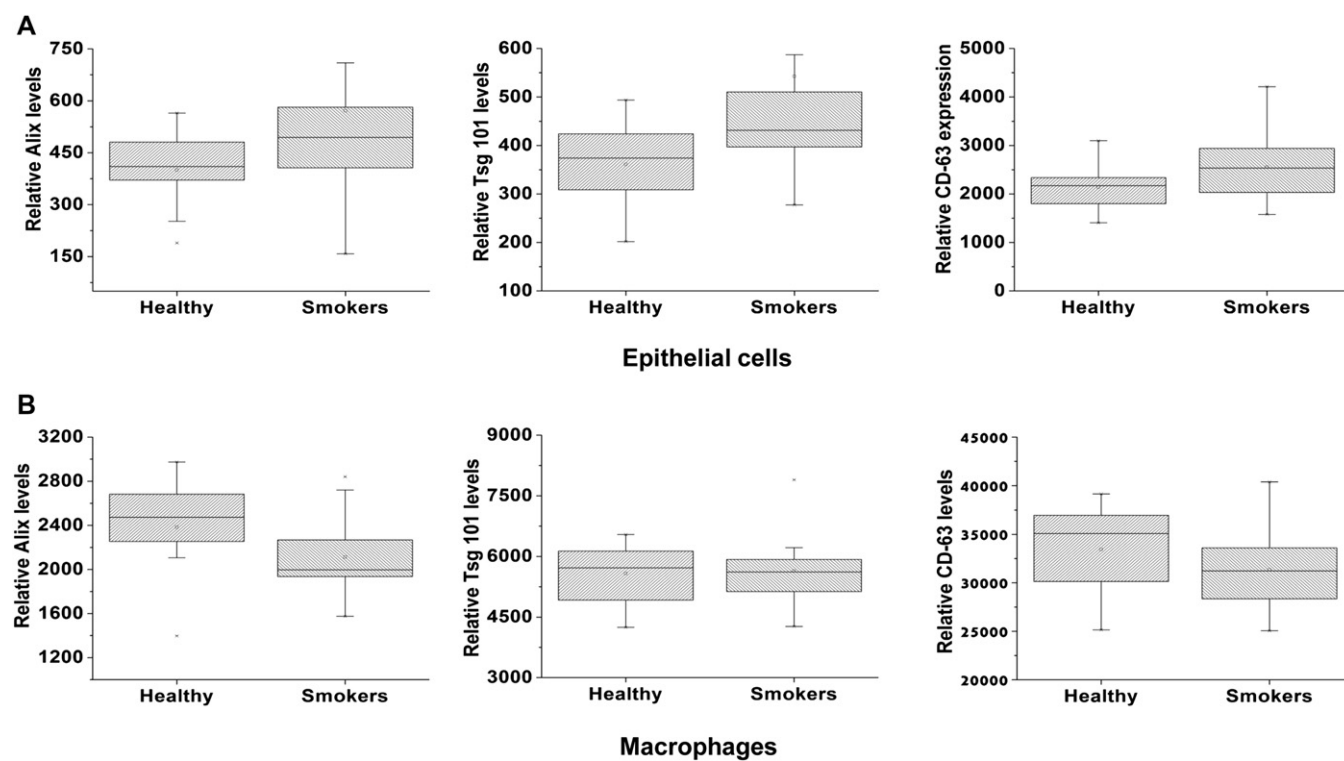


FIG E5. Major exosome-associated proteins have higher expression levels in epithelial cells (**A**) but not in alveolar macrophages (**B**) isolated from clinical subjects with chronic obstructive pulmonary disease. The data source for Fig E5, A, is GDS 534, and that for Fig E5, B, is GDS 3496.

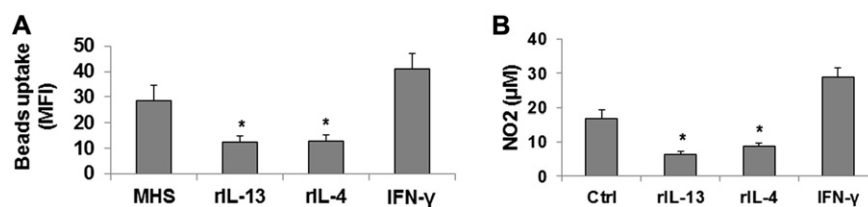


FIG E6. IL-13 and IL-4 reduce phagocytosis and nitric oxide production in macrophages. **A**, Macrophages were treated with rIL-13, rIL-4, or rIFN- γ for 24 hours before incubating them with IgG-coated fluorescent beads, and the uptake was assessed by measuring fluorescence after 24 hours of incubation. *MFI*, Mean fluorescence intensity. **B**, Macrophages were treated with rIL-13 (20 ng/mL), rIL-4 (20 ng/mL), or rIFN- γ (50 ng/mL) for 24 hours, and nitric oxide levels were measured with Griess reagent. Fig E6, *A* and *B*, are plotted as means $\pm$ SEs of 2 independent experiments * $P < .05$.

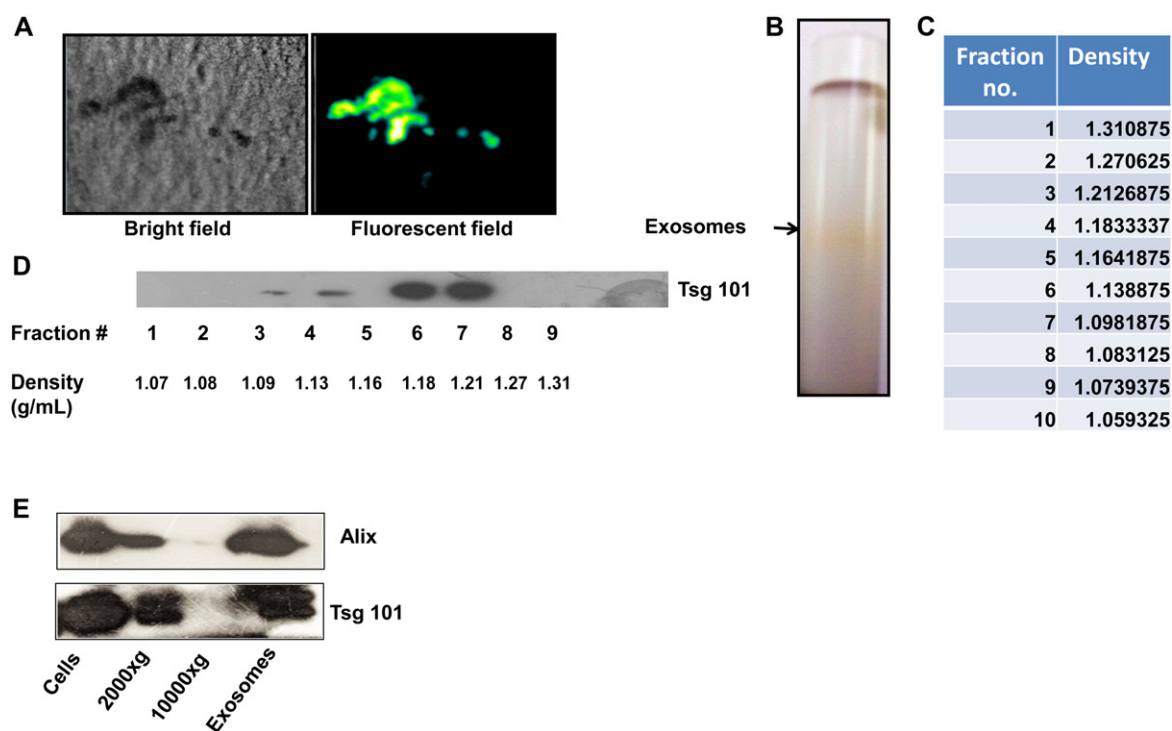


FIG E7. Characterization of exosomes from BEAS-2B cells. **A**, DIO-labeled exosome pellets were visualized under a bright field and a fluorescent field. **B**, Labeled exosome fraction on sucrose density gradient. **C**, Densities of different fractions collected from Fig E7. **D**, Tsg-101 detected in various fractions from Fig E7. **E**, Alix and Tsg-101 detected in fractions collected during the different steps of exosome isolation.

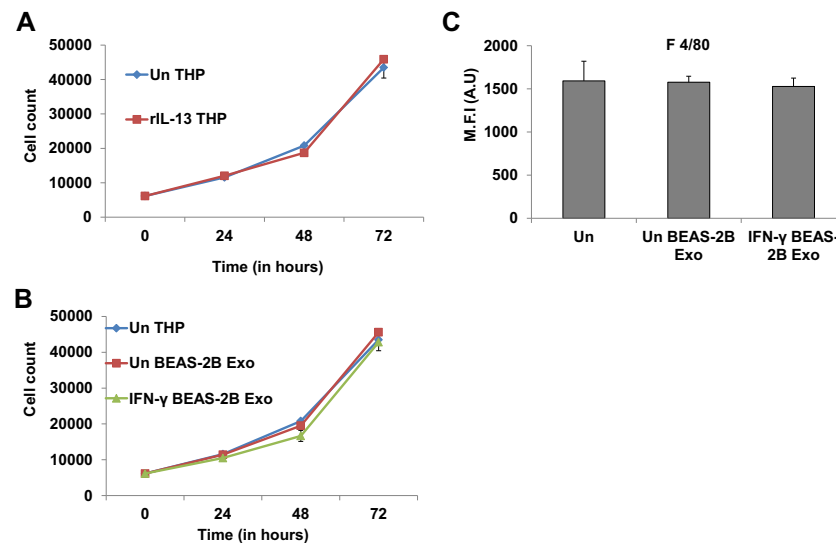


FIG E8. Exosomes derived from IFN- γ -treated BEAS-2B cells had no effect on monocyte proliferation. **A**, THP-1 cells were treated with 20 ng/mL IL-13, and the cells were counted at various time points. **B**, Proliferation of THP-1 monocytes was measured after treatment with indicated exosomes. **C**, IFN- γ -treated BEAS-2B cell-derived exosomes do not affect F4/80 expression on THP-1 cells. Cells were incubated for 72 hours with the exosomes before acquisition. *M.F.I.*, Mean fluorescence intensity. Figures are plotted as means $\pm$ SEs of 2 independent experiments.

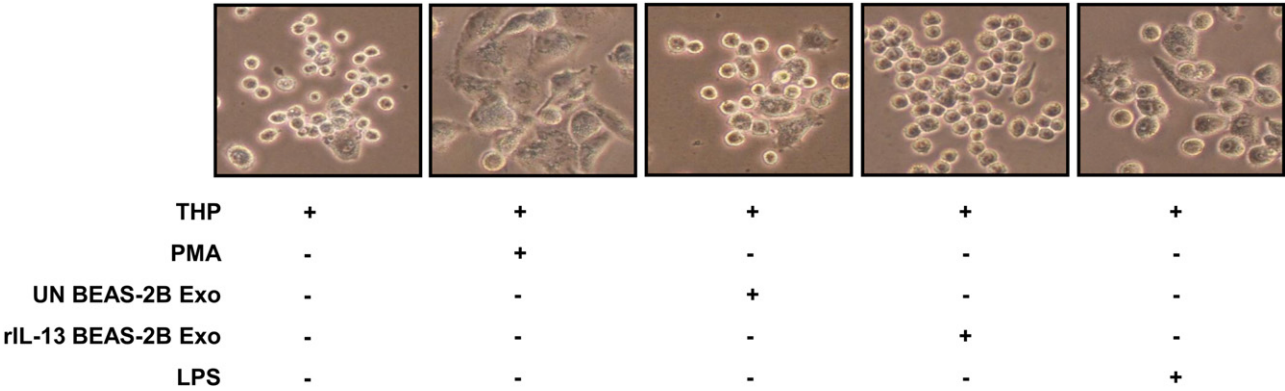
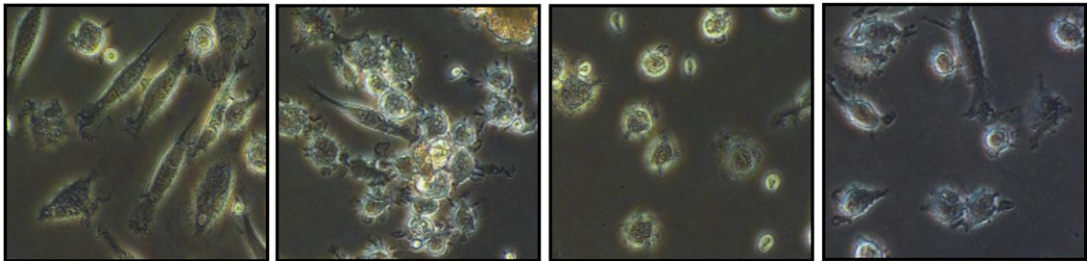


FIG E9. Exosomes derived from BEAS-2B cells treated with IL-13 and LPS induce a change in the morphology of THP-1 cells, as visualized by using microscopy. Images are representative of 3 independent experiments. Images are shown at $\times 20$ magnification. *PMA*, Phorbol 12-myristate 13-acetate.



M-CSF (20 ng/mL)	+	+	+	+
UN BEAS-2B Exo	-	+	-	-
rIL-13 BEAS-2B Exo	-	-	+	-
IFN-γ BEAS-2B Exo	-	-	-	+

FIG E10. Exosomes derived from BEAS-2B cells treated with IL-13 and IFN-γ induce a change in the morphology of primary monocytes, as visualized by using microscopy. Images are representative of 2 independent experiments and are shown at ×20 magnification.

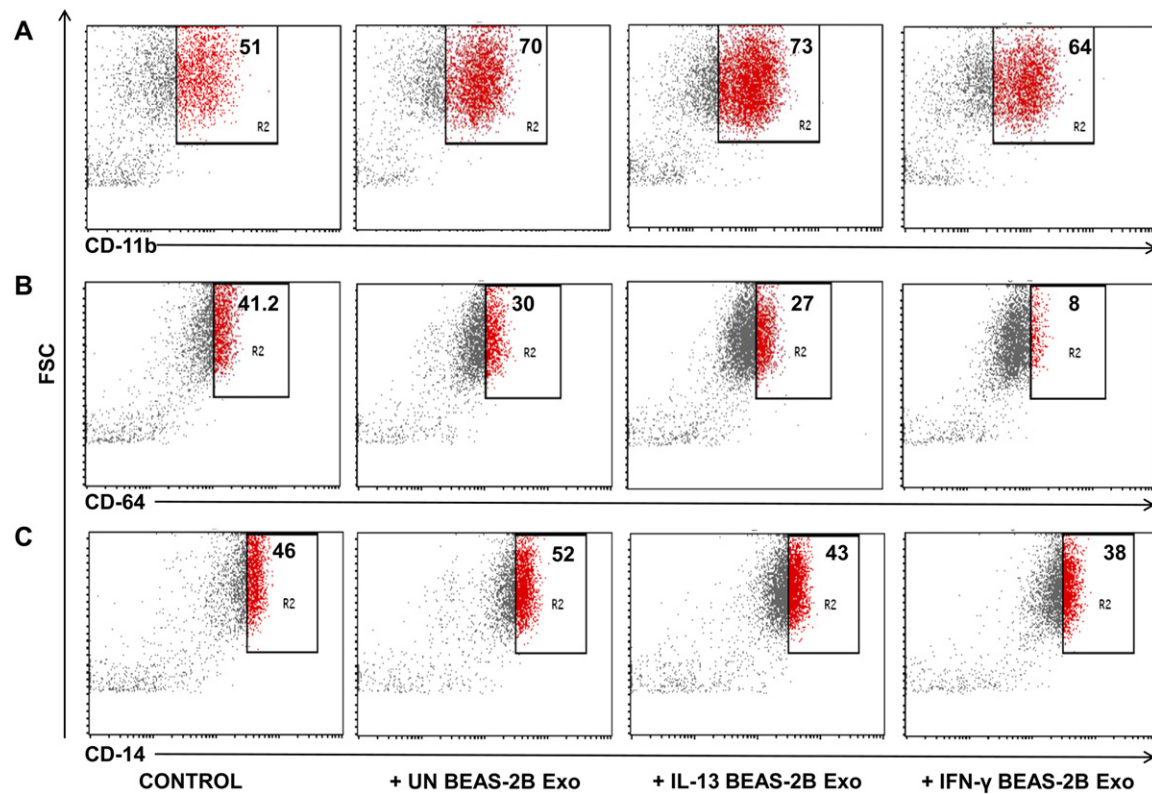


FIG E11. Cord blood–derived monocytes were cultured in the presence of macrophage colony-stimulating factor (20 ng/mL), and the effect of epithelially derived exosomes was observed on the surface expression of CD11b (A), CD64 (B), and CD14 (C) measured after 72 hours of incubation with the indicated exosomes. FSC, Forward scatter.

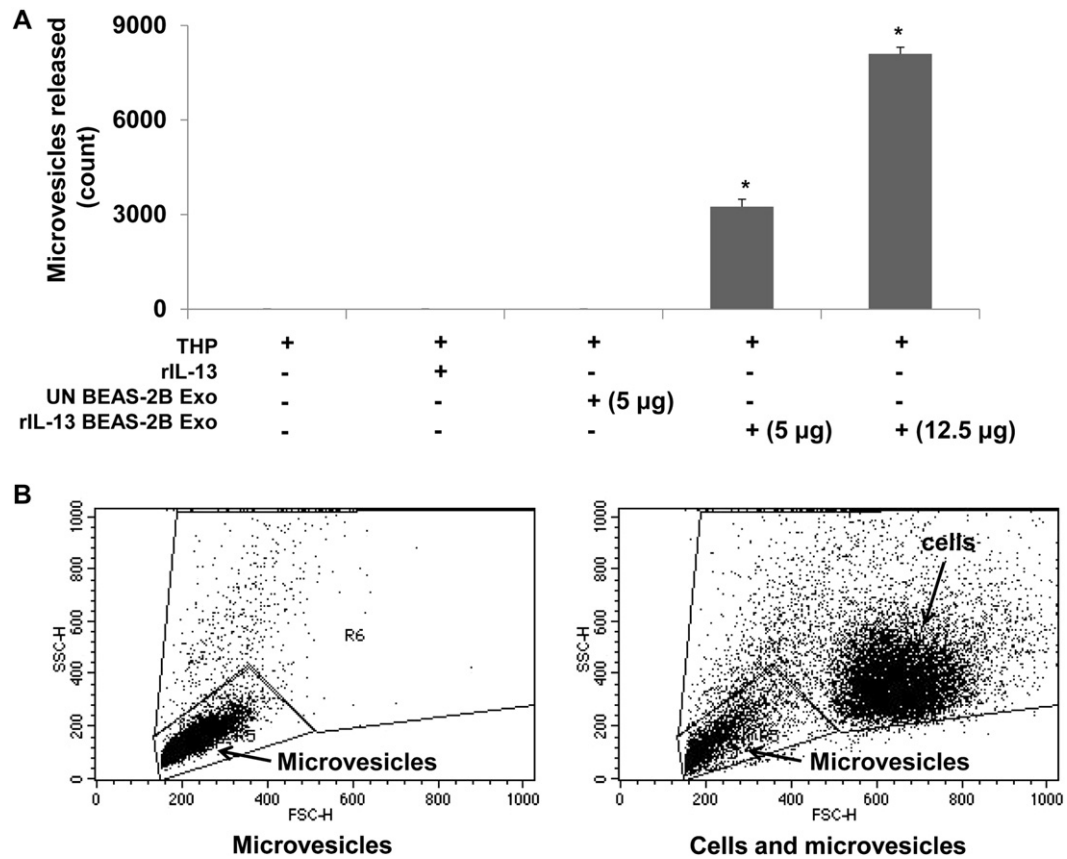


FIG E12. Exosomes derived from rIL-13-treated BEAS-2B cells induce microvesicle release from THP-1 cells. **A**, THP-1 cells pretreated with either IL-13-treated BEAS-2B cell-derived exosomes or with IL-13 itself were seeded in the upper chamber, and microvesicles released in the media in the lower chamber were counted with a flow cytometer. **B**, Gating schema used for differentiating microvesicles and cells. Fig E12, A, is plotted as the mean $\pm$ SE of 2 independent experiments. * $P < .05$.

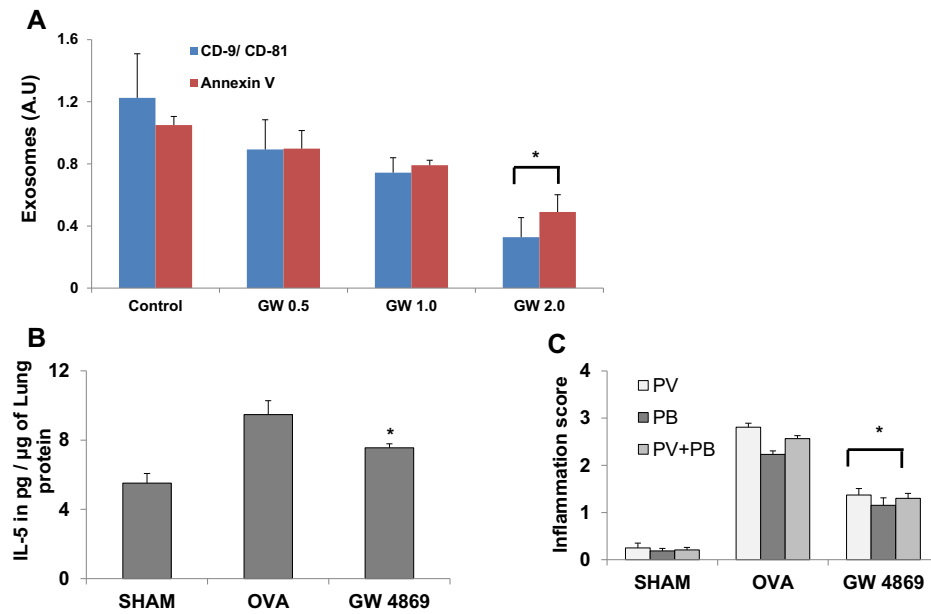


FIG E13. Reduction in exosome secretion in mouse lungs improves asthmatic features. **A**, GW4869 reduces exosome secretion *in vitro* in a dose-dependent manner. **B**, IL-5 levels measured in total lung protein. **C**, Inflammation scores in BALF. *PB*, Peribronchial; *PV*, perivascular. Fig E13, *A*, *B*, and *C*, are plotted as means $\pm$ SEs of 2 independent experiments with 4 to 6 mice in each group. * $P < .05$.