

DR GON YASUHIRO (Orcid ID : 0000-0003-0514-3054)

Article type : Original Article-Basic Mechanisms in Allergic Disease

Selective release of miRNAs via extracellular vesicles is associated with house dust mite allergen-induced airway inflammation

Yasuhiro Gon MD, PhD^a; Shuichiro Maruoka MD, PhD^{a,*}; Toshio Inoue PhD^a; Kazumichi Kuroda PhD^b; Kenji Yamagishi MD, PhD^c; Yutaka Kozu MD, PhD^a; Sotaro Shikano MD^a; Kaori Soda PhD^a; Jan Lotvall MD, PhD^d; and Shu Hashimoto MD, PhD^a

^aDivision of Respiratory Medicine, Department of Internal Medicine, Nihon University School of Medicine 30-1 Oyaguchi Kmicho, Itabashi-ku, Tokyo, 173-8610, Japan

^bDivision of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine 30-1, Oyaguchi Kami-cho, Itabashi-ku, Tokyo 173-8610, Japan

^cDepartment of Chemical Biology and Applied Chemistry, College of Engineering, Nihon University 1, Aza-Nakagawara, Tokusada, Tamuramachi, Koriyama-shi, Fukushima 963-8642, Japan

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.13016

This article is protected by copyright. All rights reserved.

^dDepartment of Internal Medicine and Department of Respiratory Medicine and Allergology, The

Sahlgrenska Academy, University of Göteborg, Box 424, SE-405 30 Gothenburg, Sweden

Keywords

Extracellular vesicles; Microvesicles; Exosomes; Asthma; House dust mite; Allergen; miRNA; Th2
immune response

***Corresponding Author:** Shuichiro Maruoka MD, PhD, Division of Respiratory Medicine,
Department of Internal Medicine, Nihon University School of Medicine, 30-1, Oyaguchi Kamicho,
Itabashi, Tokyo 173-8610, Japan. Tel: +81-3-3931-7047, Fax: +81-3-3972-2893, E-mail:
maruoka.shuichiro@nihon-u.ac.jp

Sources of funding

This work was supported by funding from the Strategic Foundational Technology Improvement
Support Operation of the Japanese Government, the Nihon University Joint Research Grant, and the
Nihon University President's Grant for Multidisciplinary Research.

Conflicts of interest

None, except that Jan Lötvall owns equity in Codiak Biosciences Inc., a company developing
exosomes as therapeutics.

Author email addresses:

Yasuhiro Gon MD, PhD, gon.yasuhiro@nihon-u.ac.jp

Shuichiro Maruoka MD, PhD, maruoka.shuichiro@nihon-u.ac.jp

Toshio Inoue, PhD, inoue.toshio@nihon-u.ac.jp

Kazumichi Kuroda PhD, kuroda.kazumichi@nihon-u.ac.jp

Kenji Yamagishi MD, PhD, yamagisi@chem.ce.nihon-u.ac.jp

Yutaka Kozu MD, PhD, kozu.yutaka@nihon-u.ac.jp

Sotaro Shikano MD, meso13012@g.nihon-u.ac.jp

Kaori Soda PhD, soda.kaori@nihon-u.ac.jp

Jan Lotvall PhD, jan.lotvall@gu.se

Shu Hashimoto MD, PhD, hashimoto.shu@nihon-u.ac.jp

Abstract

Background: MicroRNAs (miRNAs) may facilitate cell-to-cell communication via extracellular vesicles (EVs). The biological roles of miRNAs in EVs on allergic airway inflammation is unclear.

Methods: Airway secreted EVs (AEVs) were isolated from bronchoalveolar lavage fluid (BALF) of control and house dust mite (HDM) allergen-exposed HDM-sensitized mice. The expression of miRNAs in AEVs or miRNAs and mRNAs in lung tissue was analyzed using miRNA microarray.

Results: The amount of AEV increased 8.9-fold in BALF from HDM-exposed mice compared with that from sham-control mice. HDM exposure resulted in significant changes in the expression of 139 miRNAs in EVs and 175 miRNAs in lung tissues, with 54 miRNAs being common in both samples. Expression changes of these 54 miRNAs between miRNAs in AEVs and lung tissues after HDM exposure were inversely correlated. Computational analysis revealed that 31 genes, including IL-13 and IL-5Ra, are putative targets of the miRNAs upregulated in AEVs but downregulated in lung tissues after HDM exposure. The amount of AEV in BALF after HDM exposure was diminished by treatment with the sphingomyelinase inhibitor GW4869. The treatment with GW4869 also decreased Th2 cytokines and eosinophil counts in BALFs and reduced eosinophil accumulation in airway walls and mucosa.

Conclusion: These results indicate that selective sorting of miRNA including Th2 inhibitory miRNAs into AEVs and increase release to the airway after HDM exposure would be involve in the pathogenesis of allergic airway inflammation.

Key Messages

- Extracellular vesicles are actively secreted in the airway after allergen exposure.
- Th2-immune-targeted miRNAs are selectively sorted into extracellular vesicles.
- Suppressing extracellular vesicle secretion inhibits allergic airway inflammation.

Capsule summary

MicroRNAs may facilitate cell-to-cell communication via extracellular vesicles. This study demonstrated that miRNAs are secreted into airway fluid via extracellular vesicles during allergic inflammation and regulate Th2-immune responses.

Keywords

extracellular vesicle; exosome; asthma; microRNA; Th2 cytokine; house dust mite

Abbreviation

AEV, airway extracellular vesicle; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; EV, extracellular vesicle; HDM, house dust mite; miRNA, microRNA; OVA, ovalbumin; PCA, principal component analysis; qRT-PCR, quantitative reverse transcriptase PCR

Introduction

Bronchial asthma is a chronic inflammatory disorder of the respiratory tract involving recurrent wheezing, coughing, and sputum formation as symptoms and is characterized by bronchial hyper-responsiveness and remodeling of the airways^{1,2,3}. In asthma pathogenesis, interactions between various airway-structural cells, such as bronchial epithelial cells and immune cells, are believed to be responsible for the development of the local allergic inflammation^{1,4,3,5}. The full pathogenic map of asthma remains blurred, but recent findings imply that secreted extracellular vesicles (EVs), including exosomes and microvesicles, can be key regulatory factors in this disease^{6,7,8,9}.

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression and are often packaged within secreted EVs^{7,10}. EVs can be formed either as budding from the cell membrane or can be produced as secretory vesicles within the cell in multivesicular bodies. EVs can

Accepted Article

be actively taken up by recipient cells, by which they can deliver both protein and RNA species, which can influence various biological phenomena. The possibility that EVs deliver RNA interference molecules to recipient cells, such as miRNAs, is of special interest as these molecules can have vast biological influence on recipient cells ^{7, 10}.

There are a few studies concerning secreted EVs on allergic airway inflammation in bronchial asthma ^{11, 12, 13}. Kulshreshtha et al. analyzed the smallest EVs, exosomes, in the alveolar lavage fluid after OVA challenge from intraperitoneally sensitized mice with ovalbumin (OVA) and alum¹³. According to this report, exosome production in alveolar lavage fluid increased following the exposure of mice to OVA. In addition, results from this study showed that exosome release into alveolar lavage fluid decreased and that airway inflammation was ameliorated following the administration of an inhibitor of extracellular secretion of exosomes in mice. These findings indicate that intercellular communication via exosomes is integrally involved in the development of allergic airway inflammation. However, the mechanism underlying this phenomenon has remained unclear.

We hypothesized that allergic inflammation fundamentally changes the EV secretome miRNA content and therefore analyzed variations in the production of airway EVs (AEVs) and their miRNAs content in a murine asthma model.

Methods and Methods

Animal model

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained and handled under Institutional Animal Care and Use Committee-approved procedures (Nihon University School of Medicine) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Mice were matched for age and sex (7wk-old males). House dust mite (HDM) extract (Greer Laboratories, Lenoir, NC) resuspended in a 0.9% NaCl solution at a concentration of 2.0 µg/ml was administered intranasally to isoflurane-anesthetized mice in a 50-µl volume. Mice were exposed intranasally (i.n) to HDM extract on days 1, 7, and 14. A 50-µl volume of 0.9% NaCl solution was administered i.n to isoflurane-anesthetized mice as a sham control. At 2 days after the 3rd HDM extract administration, lungs were lavaged 5 times through the trachea with 1 ml of normal saline to obtain bronchoalveolar lavage fluid (BALF). After that, the lungs were perfused using saline irrigation via the right ventricle so that they were free of blood. Subsequently, the left lungs were isolated after bronchoalveolar lavage (BAL) treatment for isolation of lung tissue samples. Additionally, a dose of 1.25 mg/kg of GW4869 (Sigma-Aldrich, St. Louis, MO) or an equal volume of DMSO was intraperitoneally (i.p) injected into sham control and HDM-treated mice at day 14. The time course of the experiment is shown in Supplement data 1

Exosome purification and isolation

The EVs secreted in into the airway were termed as airway EVs (AEVs). The isolation of AEVs from BALFs, was performed using the ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA), according to the manufacturer's recommended protocol. BALFs from HDM-treated and sham-control mice were subjected to differential centrifugation at 4°C ($450 \times g$, 10 min) to remove BAL cells (mainly macrophages, lymphocytes, and granulocytes) and subsequently at $3000 \times g$ (15 min) to remove dead cells. The supernatants were filtered through 0.2- μ m filters to remove particulate material larger than 200 nm and centrifuged at $3000 \times g$ for 15 min. EVs smaller than 200 nm in size were present in these pellets.

Quantifying exosomes

Purified exosomes were quantified using a qNano nanoparticle counter (Izon Science Ltd, Christchurch).

Western blot analysis of exosomal markers

Membrane surface expression of Alix, Tsg101, and CD63 was determined by immunoblot analysis using anti Alix (Q-19, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Tsg101 (M-19, Santa Cruz), and anti-CD63 (H-193, Santa Cruz) antibodies. Exosomes obtained from BALF were lysed

with sodium dodecyl sulfate (SDS) sample buffer. Total protein (20µg) was subjected to SDS-polyacrylamide gel electrophoresis using a 10% gel under reducing conditions and was then transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were incubated with 3% bovine serum albumin Fraction V (Roche Life Science, Indianapolis, IN, USA) in PBS at 4°C for 1 h at room temperature. The PVDF membranes were incubated overnight at 4°C with primary anti-CD63, anti-Alix, and anti-Tsg101 antibodies (1:1000 dilution) in Tris-buffered saline containing 0.1% Tween-20. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated species-specific anti-rabbit Ig and anti-goat Ig (Cell Signaling Technology Inc., Beverly, MA, USA). After extensive washing of the membranes, the immunoreactive proteins were visualized using an ECL prime (GE Healthcare, Little Chalfont, UK), according to the manufacturer's recommended protocol.

Electron microscopy of isolated EVs

AEVs were resuspended in 10 µL PBS and spotted onto formvar-coated grids (200 mesh). Adsorbed exosomes were fixed in 2% (vol/vol) paraformaldehyde for 5 min at room temperature. After fixation, the exosomes were directly negatively stained using uranyl acetate. Grids were examined with a Philips CM100 electron microscope at 80 kV.

RNA extractions and microarray studies

To obtain RNA amount allowing us to conduct miRNA microarray analysis, each sampled RNAs were extracted from the pooled BALFs from 4 sham control mice or 4 house-dust-mite-treated mice. Four samples in each condition were collected in 4 separate opportunities. The extracted total RNA in AEVs was used in both microRNA array analysis and RT-PCR.

Total RNA in AEVs was extracted using the Total Exosome RNA Isolation Kit (System Biosciences, Mountain View, CA), according to the manufacturer's recommended protocol. Total RNA in lung tissues including the airway mucosa was quantified by spectrophotometry, and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Microarray analysis was performed to characterize the miRNA expression profiles of extracellular-vesicle RNAs (evRNAs) in BALF and the mRNA expression profiles of lung tissues; evRNAs were labeled using the Flash Tag RNA Labeling Kit (Genisphere, USA), according to the manufacturer's recommendation. Hybridization and washing were then performed via the Affymetrix Fluidics Station 450 and Hybridization Oven 640, under standard operating conditions. Image processing was conducted using the Affymetrix Gene Array 3000 scanner, and raw data files for each sample were analyzed using

Affymetrix miRNA QC Tool software. The Affymetrix Gene Chip miRNA 3.0 Array contains 19,724 probe sets including 1966 mature mouse miRNAs. Differentially expressed miRNAs were filtered to exclude expression changes of less than 2.0-fold ($P < 0.05$), compared with expression seen in sham-control mice. The resultant readings were quantile-normalized for each experimental condition, and a moderated *t*-test was performed, followed by false-discovery rate correction.

Preparation of the RNA libraries and sequencing EV RNA

RNA libraries were prepared using the Ion Total RNA-Seq Kit v2 (Life Technologies) per the manufacturer's recommended protocol. For library construction, the RNA samples were fragmented at 37°C for 10 min using RNase III. The fragmented-RNA was dried down to 3 µL and then combined with hybridization reagents and incubated at 65°C for 10 min and 30°C for 5 min. Ligation reagents were then added and the samples were incubated at 30°C for 1 h. After ligation, reverse transcription was performed. Briefly, reverse transcriptase (RT) master mix was added to the samples, tubes were incubated at 70°C for 10 min, samples were snap-cooled on ice, the RT enzyme was added, and the samples were incubated at 42°C for 30 min. cDNA from the RT reaction was purified using the kit's clean-up module containing magnetic beads (5 µL per well of a 96 well plate) and eluted in 12 µL of

nuclease-free water. Six microliters of the purified cDNA was combined with PCR primers and Platinum PCR SuperMix High Fidelity reaction mix. The reaction mixture was then placed in a thermocycler and amplified using the following protocol: 94°C for 2 min; 2 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 30 s; and 16 cycles of 94°C for 30 s, 62°C for 30 s, and 68°C for 30 s; and 68°C for 5 min. Once the thermocycling was complete, reactions were stored on ice until purification. The amplified DNA (final library) for each sample was purified using the kit's clean-up module containing magnetic beads (5 µL per well of a 96-well plate) and eluted in 15 µL of nuclease-free water. Final libraries were stored on ice for the short term or at -20°C for long-term storage. To assess the yield and size distributions, 1 µL of the library was run on an Agilent DNA High Sensitivity chip (Agilent). The molar concentration of the library was determined with Agilent 2100 Bioanalyzer Instrument Expert software and used to dilute libraries to an appropriate concentration for sequencing. Sequencing of each sample was performed on the Ion Torrent PGM instrument using 318 chips (11,000,000 wells per chip) and the protocol provided in the Total evRNA and Protein Isolation Kit (Invitrogen) with 500 flows (125 cycles).

Quantitative reverse transcriptase PCR (qRT-PCR) analysis of mRNA and miRNA expression

qRT-PCR was performed to study expression of let-7a-5p, mir-702-5p, mir-762, mir574-3p, mir-574-5p, mir-1827, mir-346, and mir-191-5p. Real-time PCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Denmark). Real-time PCR was performed using the SYBR® Green Master Mix Kit™ (Exiqon, Denmark) and specific primers obtained from Exiqon (Product Nos. 205727, 206999, 205476, 204365, 206999, 205463, 205158, 204663, and 204306; Exiqon, Denmark). The primer sequences used for qRT-PCR are shown in Supplement data 2. The expression levels of each miRNA in EVs relative to UNISP6 (for EVs) or miR191 (for lung tissues) were determined using the $2^{-\Delta\Delta Ct}$ method.

miRNA-RNA target prediction

To identify putative targets of miRNAs, we used Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems; <http://www.ingenuity.com>), which could be used to identify putative targets for input miRNA(s) and integrate with our mRNA microarray profile data. Before starting the analysis, the confidence level was set to “highly predicted” and “experimental observed,” and miRNA targets were predicted by an integrated database, including miRecords, Tarbase, and TargetScan Human. Next, highly predicted targets were matched and paired with

mRNA expression data by the expression-pairing function of the IPA software. The results mainly provided us biological functions and canonical pathways associated with our data and were generated automatically using the core-analysis option of the IPA software.

Statistical analysis

Data are presented as mean $\pm$ S.D. Student's *t*-test was used to analyze differences between 2 samples, and differences were considered statistically significant at $p < 0.05$. One-way ANOVA was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), Genespring GX12 software (Agilent Technologies, Santa Clara, CA), or the Partek® Genomics Suite 6.5 software program (Partech, Munster, Germany).

Results

Characterization of AEVs

C57/B6J mice were sensitized with HDM extract on days 1, 7, and 14. AEVs were collected by alveolar lavage from mice treated with HDM extract or sham-control mice at day 17. We analyzed vesicle sizes and morphological differences between AEVs from HDM-treated mice and sham-control mice by transmission electron microscopy (Figure 1A). Electron microscopic imaging using negative staining with uranyl acetate confirmed the presence of a lipid bilayer structure in

AEVs from the mice. The size of vesicles observed by electron microscopy was 50–200 nm in

diameter, which is compatible with previously reported sizes of exosomes and microvesicles^{14, 7, 10}.

We quantified AEV particle size distributions using qNano equipment, as described in the Materials and Methods section. Representative distributions for sham-control or HDM-treated-mouse-derived AEVs are shown in Figure 1B. In sham-control and HDM-treated mice, the major peak of particle size distribution fell between 30 and 100 nm. The second most common distribution in both samples was between 100 and 150 nm. No differences in size distribution in AEVs were observed between HDM-treated and sham-control mice (Figure 1B). However, the number of AEVs was increased by 8.5-fold in HDM-treated mice compared with that in sham-control mice ($P < 0.001$, Figure 1B).

AEVs obtained from BALFs in our model were expected to contain a highly enriched mixture of exosomes and microvesicles. To confirm this possibility, AEVs were immunostained with antibodies against exosome markers, including CD63, Tsg-101, and Alix^{15, 7, 7}. We compared CD63, Tsg-101, and Alix expression in AEVs between HDM-treated mice and sham-control mice to determine whether expression changes in exosomes from AEVs occur in airway lumen after HDM allergen exposure (Figure 1C). Western blot analysis revealed that expression of these exosome markers markedly increased after HDM allergen exposure.

This article is protected by copyright. All rights reserved.

Next, RNA was extracted from AEVs from HDM-treated or sham-control mice. Figure 1D shows electropherogram patterns of the extracted RNAs as determined by capillary electrophoresis. Peaks of ribosomal RNAs were not seen in total RNAs extracted from AEVs of either HDM-treated or sham-control mice. Electropherograms showed that the AEVs contained small RNAs (approximately >500 bp), including RNAs within the size range of miRNAs (Figure 1E).

Expression profiles of EV and lung-tissue miRNAs

Differences in AEV miRNA expression between HDM-treated and sham-control mice were analyzed using the Affymetrix GeneChip miRNA 3.0 microarray and Partek software (Supplement data 3). To obtain RNA amount for conducting miRNA microarray analysis, each RNAs were extracted from the pooled BALFs from 4 sham control mice or 4 house-dust-mite-treated mice. To obtain RNA amount for conducting miRNA microarray analysis, each RNA samples (sham 1~4 or HDM1~4) were extracted from the pooled BALFs from 4 sham control mice or 4 house-dust-mite-treated mice (Figure 2). The heat map and cluster analysis of AEV miRNA expression patterns in BALFs from both experimental groups (sham 1~4 or HDM1~4) is shown in Figure 2A. For all pooled samples, the clustering results matched their treatment (Figure 2A). Of 1966 murine mature and premature miRNAs analyzed with the Affymetrix GeneChip miRNA 3.0 array, 85 miRNAs showed significant

Accepted Article
differences ($P < 0.05$; ≥ 1.5 -fold expression increase or decrease) between AEVs from HDM-treated and sham-control mice. Of these, 57 miRNAs showed significantly increased and 28 showed significantly decreased expression (Figure 2B). Principal component analysis (PCA) of each pooled sample from HDM-treated and sham-control mice was shown the distinct differences between the treatment groups (Figure 2C).

Differences in miRNA expression in lung tissues including the airway mucosa between HDM-treated and sham-control mice were analyzed using the Affymetrix GeneChip miRNA 3.0 microarray (supplement data 4). Heat map and cluster analysis of miRNA expression patterns in lung tissues from 4 HDM-treated mice and 4 sham-control mice (randomly collected a lung tissue sample from 4 lung tissues using pooled sample of Figure 2) is shown in Figure 3A. Of 1966 murine miRNAs analyzed with the Affymetrix GeneChip miRNA 3.0 microarray, 79 miRNAs showed significant ($P < 0.05$), ≥ 1.5 -fold differences in expression between the lung tissues from HDM-treated and sham-control mice. Of these, the expression of 30 miRNAs was significantly increased and that of 49 miRNAs was significantly decreased (Figure 3B). PCA analysis shown that distinct differences in the miRNA expression patterns were noted between the two groups (Figure 3C). Fifty-four miRNAs were found in common between the 85 miRNAs showed alterations in expression in AEVs after HDM

treatment and the 79 miRNAs showed alterations in expression in lung tissues after HDM treatment ($p = .0001$, Fisher's exact test) (Figure 4, upper panel).

Next, we focused on the 54 common miRNAs. Of the 34 miRNAs elevated in ASMs, 25 miRNAs (71.4%) showed reduced expression in lung tissues (Figure 4 lower). Of 20 miRNAs downregulated in ASMs, 13 (65%) miRNAs were upregulated in lung tissues (Figure 4, lower). The differences in miRNA expression level changes in AEVs and lung tissues were negatively correlated, with $R = -0.6147$ and $P > 0.001$ (Figure 4, lower).

Target analysis of key miRNA–mRNA interactions in the murine asthma model

In general, miRNAs modulate gene expression through both mRNA degradation and translational repression mechanisms^{16, 17, 18}. To identify potential target transcripts of miRNAs showing increased in EVs and decrease in the lung tissue, a systematic analysis of interactions between miRNAs and target mRNAs based on microarray data was performed to evaluate the potential functional roles of miRNAs. We analyzed differences in mRNA expression in lung tissues harvested from the HDM-treated and sham-control groups, using a genome-wide microarray. The Partech-based heat map and the cluster analysis of mRNA expression patterns in lung tissues from 4 mice at 17 days post-HDM administration and 4 sham-control mice are shown in (Supplement data 5).

Distinct differences in mRNA expression patterns were found between the 2 groups. A total of 3006

This article is protected by copyright. All rights reserved.

mRNAs were significantly altered ($P < 0.05$) in lung tissues from the HDM-treated group compared with those from the sham-control group, of which 1100 mRNAs were increased and 1906 mRNAs were decreased. Top 100 up or down regulated genes were shown in Supplement data 6.

Having performed miRNA and mRNA microarray profiling for the same samples, we were able to analyze correlations between the altered miRNAs and mRNAs identified. miRNA–mRNA interaction analysis was conducted using IPA software and microarray-based mRNA expression data from lung tissue. Presumed target mRNAs of these miRNAs were identified using IPA software with the “highly predicted” and “experimental observed” criteria selected for analysis. Thirty-one miRNA:mRNA pairs (3 miRNAs and 31 mRNAs) were identified by this method (Table 1). Of the miRNAs expressed at elevated levels in EVs and at decreased levels in lung tissues, 31 mRNAs with possible targets identified with IPA software were subjected to pathway analysis with IPA to study associated canonical pathways. The IPA pathway analysis results revealed the top functions of these 31 target genes. The most affected biological processes for bronchial asthma (BA) included “T-helper cell differentiation,” “granulocyte adhesion and diapedesis,” “leukocyte extravasation signaling,” and “airway inflammation in asthma” (Table 2). Among these miRNAs in the AEVs, mir-346 was predicted to directly target the Th2 cytokine, IL-13 (Table 2).

Validation of microarray data by qRT-PCR analysis

To validate the microarray results, we performed qRT-PCR analysis on the samples. The expression data obtained by qRT-PCR analysis were comparable to the results observed in the microarray analysis. The expression of miR-346, miR-1827, and miR-574-5p was consistently downregulated in the pooled RNA samples from HDM-exposed mice compared with that in sham-control mice, in agreement with the microarray results obtained using lung tissues, while these miRNAs were consistently upregulated in EVs obtained from HDM-exposed mice compared with those obtained from sham-control mice. Figure 5 shows the fold-changes observed in real-time qRT-PCR ($2^{-\Delta\Delta C_t}$) and miRNA microarray experiments. Consistency between the microarray and real-time qRT-PCR experiments confirmed the reliability of the results.

Effects of reducing exosome secretion by GW4869 on allergic airway inflammation

The results described above suggested that Th2-immune targeted miRNAs are selectively released into the airway lumen via AEVs during allergic airway inflammation. We hypothesized that the reduction of exosome secretion in the airways may suppress allergic airway inflammation. GW4869, an inhibitor of neutral sphingomyelinase (nSMase), has been reported to significantly decrease the release of exosomes from cells^{13, 19-25}. To test our hypothesis, we used GW4869 in the murine asthma model. The number of AEVs and their RNA contents in BALFs after HDM exposure were diminished

by GW4869 pretreatment (Figure 6A). The quantity of miR-346, miR-1827, and miR-574-5p in

AEVs were also diminished by GW4869 pretreatment (Figure 6B). GW4869 pretreatment decreased the number of eosinophils in BALFs (Figure 7A) and inhibited the accumulation of eosinophils in the airway wall and mucosa (Figure 7B). GW4869 pretreatment significantly suppressed the production of IL-4 and IL-13 in BALFs (Figure 8). GW4869 pretreatment showed a tendency to suppress IL-5 production, but the difference did not reach statistical significance (Figure 8).

Discussion

The goal of this study was to understand the roles of AEVs and AEV-associated miRNAs induced by HDM allergens during allergic inflammation. Using a well-characterized mouse asthma model that leads to a Th2 immune response in the airway, we evaluated the characteristics and changes of AEVs upon exposure to HDMs. We also explored the whole-transcriptome miRNA expression profiles in AEVs, and studied the association between these miRNAs and Th2-type airway inflammation.

We found that AEVs were significantly upregulated in HDM-treated mice, suggesting that secretion of EVs into the airway is enhanced in response to HDM exposure. Western blot analysis revealed that the expression of several exosome-associated proteins, such as ALIX, TSG101, and CD63, increased in the AEVs after HDM allergen exposure, also suggestive of increased exosome

This article is protected by copyright. All rights reserved.

release. Although they used a different allergen and sensitization method, Kulshreshtha et al.

previously reported that the number of exosomes in alveolar lavage fluid markedly increased after

allergen exposure in a murine asthma model intraperitoneally sensitized with OVA ¹³. In addition,

Eltom et al. reported that BALF EVs were released in response to lipopolysaccharide. Taken together,

these findings suggest that increased AEV secretion occurs in response to allergens and pathogens ²⁶.

Although the determination of cells actively secreting AEVs could not be accomplished in this study,

in a previous report, CD63-positive bronchial cells and macrophages produced granular vesicles in an

OVA-sensitized murine asthma model ¹³, suggesting increased exosome secretion by these cells under

allergic inflammatory conditions.

EVs are known to be enriched in small RNA species, including miRNAs, which are functional in recipient cells ^{27, 28, 10, 29}. Previous studies have identified the cellular machinery that

regulates the active sorting of specific sets of miRNAs into exosomes ^{30, 31, 32, 30}. In this study, we

compared the miRNA expression profiles of secreted AEVs and lung tissues. We found that the

miRNA changes after HDM exposure showed opposite expression patterns in AEVs and lung tissues

(Figure 4). We observed that the sorting of miRNAs into EVs and their secretion into the airway

space after HDM exposure resulted in decreased levels of these miRNAs in lung tissues. Conversely,

miRNAs that were decreased in AEVs were increased in lung tissues (Figure 9). It has been reported

that some miRNAs are released in greater abundance than other miRNAs, suggesting potential roles in signaling³². Alternatively, the release of miRNAs in EVs may reflect a trash-disposal mechanism, in which cells release damaged or unneeded cellular components into the environment³³.

The miRNA mechanism of action involves suppressing the expression of target genes by destabilizing and degrading mRNAs, as well as inhibiting translation^{16, 17, 18}. A recent RNA sequencing and bioinformatics modeling study showed that the sorting of miRNAs into exosomes is directly controlled by the relative abundance of their target mRNAs in the cell that produces them³⁴. To

explore the biological roles of AEV miRNAs in regulating expression changes, we focused on miRNAs that were upregulated in AEVs and downregulated in lung tissues, and employed a bioinformatic analysis to identify several cellular processes that were significantly targeted by miRNA-modulated mRNAs. At present, IPA is one of the most reliable programs for comprehensive gene expression analysis and the prediction of molecular interactions and signaling pathways^{35, 36, 37}.

We used the miRNA–mRNA interaction analysis function of IPA to predict mRNAs in lung tissues that are targeted by upregulated AEV miRNAs, and identified 31 miRNA–mRNA interaction pairs.

To investigate the possible roles of these 31 mRNAs in allergic diseases, these genes were analyzed with IPA. We found 10 canonical pathways that were predicted to be of highest relevance, including pathways associated with allergic inflammation, such as “T-helper cell differentiation,” “granulocyte

adhesion and diapedesis,” “leukocyte extravasation signaling,” and “airway inflammation in asthma.”

This finding suggests that miRNA secretion through AEVs is involved in regulating allergic airway inflammation.

Our results suggest that increased exosome secretion of miRNAs, which can regulate several inflammatory pathways, may play an active role in the pathogenesis of allergic airway inflammation. To study this possibility, we investigated the effects of inhibiting exosome release on allergic airway inflammation. Results from previous studies have shown that exosome secretion can be prevented by the inhibition of Neutral Sphingomyelinase 2 (nSMase2), a key regulatory enzyme generating ceramide from sphingomyelin^{13, 19-25}. Decreased nSMase2 activity has been reported upon treatment with the chemical inhibitor GW4869³⁸⁻⁴⁰. We demonstrated that pretreatment with GW4869 markedly suppressed AEV secretion. This finding indicates that increased exosome secretion is dependent on the ceramide/sphingomyelin pathway. The finding also demonstrates that most AEVs induced by HDM allergens are exosomes. It was previously reported that GW4869 suppresses exosome secretion in alveolar lavage fluid and allergic inflammation in OVA-sensitized mice¹³, a result consistent with our observations. In our model, GW4869 pretreatment significantly reduced HDM allergen-induced Th2 cytokine production. In particular, IL-13 production into the BALF was almost completely inhibited by GW4869 pretreatment.

This article is protected by copyright. All rights reserved.

IL-13 plays critical roles in Th2-mediated immune responses, allergic airway inflammation, and asthma^{41, 42}. In our miRNA–mRNA interaction analysis, miR-346, which has been reported to play roles in gut mucosal inflammation, rheumatoid arthritis, and acute respiratory syndrome^{43, 23, 44}, was highly predicted to target IL-13. However, the roles of mir-346 in allergy and asthma have not been previously reported.

Our study has a few limitations: First, we only investigated a single asthma model, and only measured AEV changes at one time point; we focused only on the role of exosomes in the BALF, and we used a computational prediction of miRNA-mRNA binding. This method has the potential to be error-prone due to non-canonical binding and general inadequacy of current prediction algorithms. While exosomes secreted into the respiratory tract appear to play diverse roles in the process of allergic inflammation, due to these limitations, we were unable to demonstrate their precise roles. However, our results strongly suggest that the secretion of miRNAs via EVs (including exosomes) into the airway lumen may be an important mechanism promoting the pathogenesis of allergic airway inflammation.

In summary, we have demonstrated that HDM stimulation promotes the secretion of exosomes containing increased levels of miRNAs that function to inhibit molecules involved in Th2 inflammation, such as the IL-5 receptor and IL-13. These miRNAs had reduced expression in lung tissues, whereas miRNAs that were reduced in the exosomes were increased in the lung tissue, demonstrating an inverse correlation. The administration of GW4869, an inhibitor of exosome secretion, reduced the number of exosomes in the BALF and reduced the expression of miRNAs that inhibit Th2 inflammation, resulting in eosinophilic airway inflammation. These results suggest that intracellular Th2 inflammation is promoted by the release of miRNAs with Th2 inflammation-suppressing action out of the cells through exosomes. Thus, the increase in the expression of these molecules induces allergic inflammation (Figure 9). The results also indicate that AEVs are potentially useful biomarkers for predicting lung pathology.

Acknowledgements

This work was supported in part by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (C) Grant Number 15K09196, Nihon University President's Grant for Multidisciplinary Research and the Strategic Research Foundation for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan Grant Number S1091023.

Authors' contributions

YG had full access to the data and contributed to the study concept and design; performed experiments; participated in the interpretation, writing, and drafting of the submitted manuscript; performed critical review for important intellectual content; and gave approval for the final version to be published. SM, TI, KK, KY, DK, YS, SS, KS, ET, and IT contributed to the study concept and design, animal experiments, data collection and interpretation, and the critical review for important intellectual content. YK contributed to performing the bioinformatics analyses. JL contributed to the study by helping with the conceptual design of the study and with the finalization of the manuscript. SH and SM contributed to the study concept and design, participated in the critical review for important intellectual content, and evaluated the accuracy of the data analysis. All authors read and approved of the final manuscript.

Figure legends

Figure 1

Characterization of AEVs in HDM-induced murine asthma model. (A) AEVs from HDM-treated or sham-control mice were characterized morphologically by transmission electron microscopy. (B) Size analysis confirmation of AEVs. Representative particle diameters obtained from BALFs in HDM-treated and sham-control mice are shown. (C) Increase numbers of AEVs from HDM-treated mice. (D) Surface exosome markers on AEVs from HDM-treated or sham-control mice. (E)

Electropherogram patterns of the extracted RNAs extracted from AEVs from HDM-treated or sham-control mice.

Figure 2

Summary of genome-wide miRNA profiles of AEVs in HDM-treated or sham-control mice. (A) A hierarchical clustering analysis based on the expression patterns. Pooled samples from HDM-treated mice are yellow bar, and from sham control mice are blue bar. Each pooled sample were corrected from 4 mice. Gene-expression intensities were standardized between -2.0 (blue) and $+2.0$ (red). (B) Volcano plot showing gene expression levels in AEVs from HDM-treated vs. sham-control mice. (C) PCA mapping of gene expression in AEVs from HDM-treated vs. sham-control mice. Sham-control mice (blue), and HDM-treated mice (Red).

Figure 3

Summary of genome-wide miRNA profiles in lung tissues from HDM-treated or sham-control mice. (A) A hierarchical clustering analysis based on the expression patterns. HDM-treated mice are yellow bar, and sham control mice were blue bar. Gene-expression intensities were standardized between -2.0 (blue) and $+2.0$ (red). (B) Volcano plot showing gene expression levels in lung tissues from HDM-treated vs. sham-control mice. (C) PCA mapping of gene expression in lung tissues from

HDM-treated vs. sham-control mice. Sham-control mice are blue, and HDM-treated mice are Red.

Four mice were used in each group.

Figure 4

Negative correlation of altered miRNA expression levels in both AEVs and lung tissues after HDM exposure. Venn diagram of genes differentially expressed between AEVs and lung tissues after HDM exposure (upper panel). The p-values of the overlaps was calculated by Fisher's exact test. Negative correlation of altered miRNA expression levels between AEVs and lung tissues after HDM exposure (lower panel). Spearman's correlation test: $R = -0.6168$, $P < 0.0001$.

Figure 5

Validation of miRNA microarray results by qRT-PCR. qRT-PCR validation in lung tissues and EVs treated with or without HDM was performed for miR-346, miR-1827, miR-574-5p, and miR-577-5p.

Fold-changes in expression measured with the miRNA microarray are shown as log2 values (left y-axis, black bars). Fold-changes in expression measured by qRT-PCR were determined using the $2^{-\Delta\Delta C_t}$ method, and all miRNA expression values were normalized (right y-axis, white bars). Error bars represent the standard deviation of the mean (SD).

Figure 6

Effect of GW4869 on the reduction of EV secretion and EV miRNAs. A. GW4869 reduced AEV secretion in HDM-treated and sham-control mice (n = 12/group). B. Expression of mirR-346, miR-346, and miR-1827 in HDM-treated mice (n = 12) or HDM + GW4869-treated mice (n = 12) were examined by qRT-PCR.

Figure 7

Effect of reduced exosome secretion in mouse lungs on allergic airway inflammation. A. Hematoxylin and eosin-stained lung sections depicting the extent of airway inflammation under various experimental conditions. B. BALFs were harvested from HDM-treated (n = 12) or sham-control mice (n = 12), and total cells, lymphocytes, neutrophils, and eosinophils in BALFs were counted. Eosinophil counts, but not neutrophil and lymphocyte counts, were significantly reduced by GW4869 pretreatment. Results are expressed as means $\pm$ SE. * $P < 0.05$, ** $P < 0.01$. NS, not statistically significant.

Figure 8

Effect of reducing exosome secretion on Th2 cytokine production. BALFs were harvested from HDM-treated (n = 12) or sham-control mice (n = 12). The concentrations of IL-4 (A), IL-5 (B), and IL-13 (C) in BALFs are shown. Results are expressed as mean $\pm$ SE. * P < 0.05, ** P < 0.01.

Figure 9

A schema for proposed roles of airway secreting extracellular vesicles (AEVs) in allergic airway inflammation. Selective sorting of miRNA including Th2 inhibitory miRNAs into AEVs and increase secretion to the airway after HDM exposure are negatively correlated with their miRNA changes in cells of the airway mucosa, suggesting that the involvement in the pathogenesis of allergic airway inflammation.

Table 1

Predicted targets of significantly upregulated miRNA in AEVs in response to HDM exposure. All targets are predicted by integrated miRNA–mRNA analysis using IPA software, based on the TargetDcan and miRanda databases. Each target shows a statistically significance and an inverse correlation of expression compared with the miRNAs of interest ($P < 0.05$).

Symbol	target mRNA	Fold change
miR-1827	ADAMTS4	1.341
	BRIP1	1.265
	CD209	2.469
	CD3G	1.89
	CLSPN	1.342
	EGLN3	1.766
	FOXP3	1.336
	HORMAD2	1.223
	IGF1	2.543
	OCSTAMP	1.51
	SCML4	1.401
	SLA2	1.324
	SPN	1.615

miR-346	ARNT2	1.325
	CACNA1I	1.209
	EGLN3	1.766
	F7	1.607
	IL13	1.345
	LHX6	1.403
	MMP24	1.333
	NODAL	1.333
	PSAP	1.349
	SCML4	1.401
	SH3PXD2B	1.552
	SP7	1.254
	UHRF1	1.302
	ZNF831	1.323
miR-574-5p	CD84	2.124
	IL5RA	1.243
	MDGA1	1.289
	MS4A7	2.474

Table 2

Canonical pathways regulated by predicted target genes of miRNAs with altered expression in AEVs following HDM exposure. IPA software was used to identify canonical pathways and related target genes of significantly altered miRNAs (let-7a-5p, miR-702-5p, miR-762, miR-574-3p, miR-574-5p, miR-1827, miR-346, and miR-191-5p) in AEVs from HDM-treated vs. sham-control mice.

Ingenuity Canonical Pathways	Target genes in the respective pathways	miRNAs associated with the mRNA targets
T Helper Cell Differentiation	FOXP3, TNFRSF1B, IL13	72
Granulocyte Adhesion and Diapedesis	MMP25, THY1, TNFRSF1B, MMP24	182
Leukocyte Extravasation Signaling	SPN, MMP25, THY1, MMP24	210
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	IGF1, SP7, TNFRSF1B, ADAMTS4	250
HIF1 α Signaling	MMP25, EGLN3, MMP24	112
Inhibition of Matrix Metalloproteases	MMP25, MMP24	40
Airway Inflammation in Asthma	IL13	6
OX40 Signaling Pathway	CD3G, TNFRSF4	97
Bladder Cancer Signaling	MMP25, MMP24	97
Crosstalk between Dendritic Cells and Natural Killer Cells	CD209, TNFRSF1B	121

Supplement data 1

Timeline of the experimental protocol. Mice were intranasally sensitized 2 times with HDM extract once a week. On day 14, mice were challenged by intranasal injection with HDM extract. BAL treatment was performed at 48 h after the last challenge.

Supplement data 2

Sequences of the mature small RNA molecules investigated. All sequences are in shown in 5'–3' orientation.

Supplement data 3

FC and *P* values for differentially expressed miRNAs between miRNAs in AEVs obtained from HDM-treated and sham-control mice.

Supplement data 4

FC and *P* values for differentially expressed miRNAs in lung tissues between HDM-treated and sham-control mice.

Supplement data 5

Summary of genome-wide mRNA profiles of lung tissues in HDM-treated or sham-control mice. A hierarchical clustering analysis on the basis of the expression patterns. HDM-treated mice are yellow bar, and sham-control mice are blue bar. Gene-expression intensities were standardized between -2.52 (blue) and $+2.52$ (red).

Supplement data 6

FC and *P* values for top 100 up-regulated or down-regulated mRNAs of lung tissues in HDM-treated mice compared to sham-control mice.

References

1. Holtzman MJ, Byers DE, Alexander-Brett J, Wang X. The role of airway epithelial cells and innate immune cells in chronic respiratory disease. *Nat Rev Immunol* 2014; 14:686-98.
2. Martinez FD, Vercelli D. Asthma. *Lancet* 2013; 382:1360-72.
3. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011; 242:205-19.
4. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008; 8:193-204.
5. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol* 2014; 10:356-64.
6. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014; 14:195-208.
7. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci* 2013; 126:5553-65.

- Accepted Article
8. Zhang B, Yin Y, Lai RC, Lim SK. Immunotherapeutic potential of extracellular vesicles. *Front Immunol* 2014; 5:518.
 9. Zhou R, O'Hara SP, Chen XM. MicroRNA regulation of innate immune responses in epithelial cells. *Cell Mol Immunol* 2011; 8:371-9.
 10. Kosaka N, Yoshioka Y, Hagiwara K, Tominaga N, Katsuda T, Ochiya T. Trash or Treasure: extracellular microRNAs and cell-to-cell communication. *Front Genet* 2013; 4:173.
 11. Mazzeo C, Canas JA, Zafra MP, Marco AR, Fernandez-Nieto M, Sanz V, et al. Exosome secretion by eosinophils: A possible role in asthma pathogenesis. *J Allergy Clin Immunol* 2015; 135:1603-13.
 12. Levanen B, Bhakta NR, Torregrosa Paredes P, Barbeau R, Hiltbrunner S, Pollack JL, et al. Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. *J Allergy Clin Immunol* 2013; 131:894-903.
 13. Kulshreshtha A, Ahmad T, Agrawal A, Ghosh B. Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol* 2013; 131:1194-203, 203 e1-14.

- Accepted Article
14. Lasser C, Eldh M, Lotvall J. Isolation and characterization of RNA-containing exosomes. *J Vis Exp* 2012:e3037.
 15. Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J Proteome Res* 2008; 7:5157-66.
 16. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 2006; 20:515-24.
 17. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 2010; 79:351-79.
 18. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* 2011; 12:99-110.
 19. Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. *J Cell Biol* 2010; 190:1079-91.
 20. Charrier A, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, et al. Exosomes mediate intercellular transfer of pro-fibrogenic connective tissue growth factor (CCN2) between hepatic stellate cells, the principal fibrotic cells in the liver. *Surgery* 2014; 156:548-55.

- Accepted Article
21. Chen L, Charrier A, Zhou Y, Chen R, Yu B, Agarwal K, et al. Epigenetic regulation of connective tissue growth factor by MicroRNA-214 delivery in exosomes from mouse or human hepatic stellate cells. *Hepatology* 2014; 59:1118-29.
 22. Dinkins MB, Dasgupta S, Wang G, Zhu G, Bieberich E. Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* 2014; 35:1792-800.
 23. Guo BB, Bellingham SA, Hill AF. The neutral sphingomyelinase pathway regulates packaging of the prion protein into exosomes. *J Biol Chem* 2015; 290:3455-67.
 24. Ohshima K, Kanto K, Hatakeyama K, Ide T, Wakabayashi-Nakao K, Watanabe Y, et al. Exosome-mediated extracellular release of polyadenylate-binding protein 1 in human metastatic duodenal cancer cells. *Proteomics* 2014; 14:2297-306.
 25. Wang X, Huang W, Liu G, Cai W, Millard RW, Wang Y, et al. Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. *J Mol Cell Cardiol* 2014; 74:139-50.

26. Eltom S, Dale N, Raemdonck KR, Stevenson CS, Snelgrove RJ, Sacitharan PK, et al. Respiratory infections cause the release of extracellular vesicles: implications in exacerbation of asthma/COPD. PLoS One 2014; 9:e101087.
27. Villarroya-Beltri C, Baixauli F, Gutierrez-Vazquez C, Sanchez-Madrid F, Mittelbrunn M. Sorting it out: regulation of exosome loading. Semin Cancer Biol 2014; 28:3-13.
28. Lee Y, El Andaloussi S, Wood MJ. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Hum Mol Genet 2012; 21:R125-34.
29. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007; 9:654-9.
30. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun 2011; 2:282.
31. Nolte-'t Hoen EN, Buermans HP, Waasdorp M, Stoorvogel W, Wauben MH, 't Hoen PA. Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective

incorporation of small non-coding RNA biotypes with potential regulatory functions. *Nucleic Acids Res* 2012; 40:9272-85.

32. Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, et al. Selective release of microRNA species from normal and malignant mammary epithelial cells. *PLoS One* 2010; 5:e13515.
33. Johnstone RM, Mathew A, Mason AB, Teng K. Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. *J Cell Physiol* 1991; 147:27-36.
34. Squadrito ML, Baer C, Burdet F, Maderna C, Gilfillan GD, Lyle R, et al. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Rep* 2014; 8:1432-46.
35. Kramer A, Green J, Pollard J, Jr., Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* 2014; 30:523-30.
36. Ngwa JS, Manning AK, Grimsby JL, Lu C, Zhuang WV, Destefano AL. Pathway analysis following association study. *BMC Proc* 2011; 5 Suppl 9:S18.

- Accepted Article
37. Jimenez-Marin A, Collado-Romero M, Ramirez-Boo M, Arce C, Garrido JJ. Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. BMC Proc 2009; 3 Suppl 4:S6.
38. Luberto C, Hassler DF, Signorelli P, Okamoto Y, Sawai H, Boros E, et al. Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. J Biol Chem 2002; 277:41128-39.
39. Cogolludo A, Moreno L, Frazziano G, Moral-Sanz J, Menendez C, Castaneda J, et al. Activation of neutral sphingomyelinase is involved in acute hypoxic pulmonary vasoconstriction. Cardiovasc Res 2009; 82:296-302.
40. Walton KA, Gugiu BG, Thomas M, Basseri RJ, Eliav DR, Salomon RG, et al. A role for neutral sphingomyelinase activation in the inhibition of lipopolysaccharide action by phospholipid oxidation products. J Lipid Res 2006; 47:1967-74.
41. Wills-Karp M. Interleukin-13 in asthma pathogenesis. Curr Allergy Asthma Rep 2004; 4:123-31.

- Accepted Article
42. Izuhara K, Umeshita-Suyama R, Akaiwa M, Shirakawa T, Deichmann KA, Arima K, et al. Recent advances in understanding how interleukin 13 signals are involved in the pathogenesis of bronchial asthma. *Arch Immunol Ther Exp (Warsz)* 2000; 48:505-12.
43. Huang C, Xiao X, Chintagari NR, Breshears M, Wang Y, Liu L. MicroRNA and mRNA expression profiling in rat acute respiratory distress syndrome. *BMC Med Genomics* 2014; 7:46.
44. Chatzikyriakidou A, Voulgari PV, Georgiou I, Drosos AA. miRNAs and related polymorphisms in rheumatoid arthritis susceptibility. *Autoimmun Rev* 2012; 11:636-41.

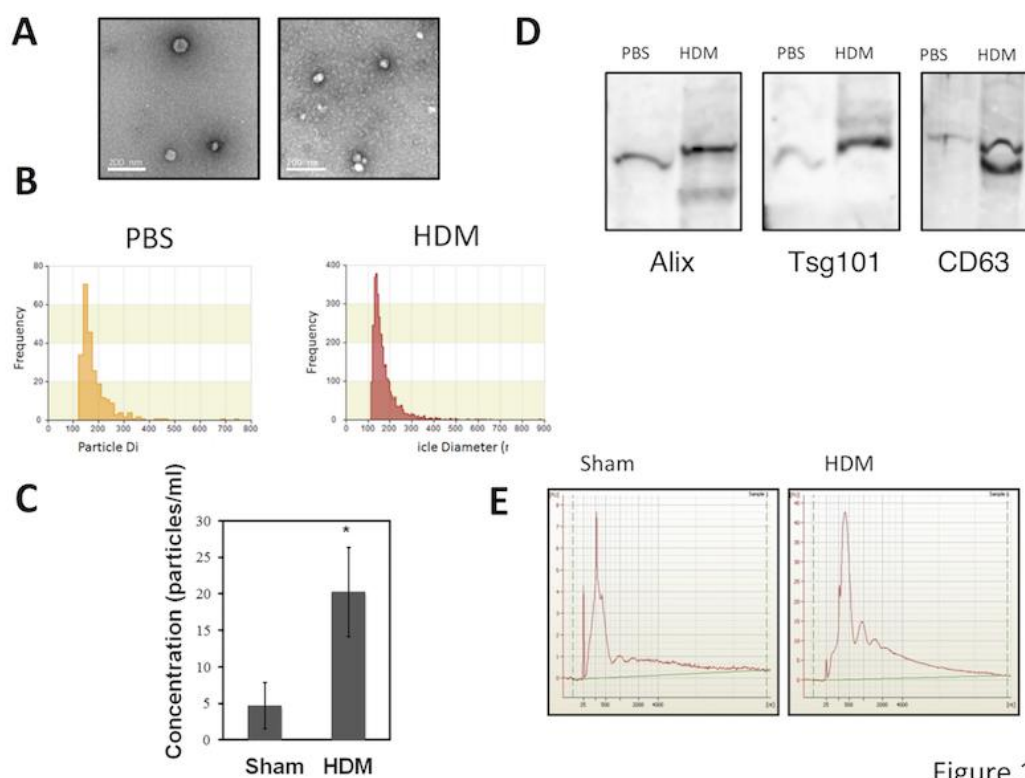
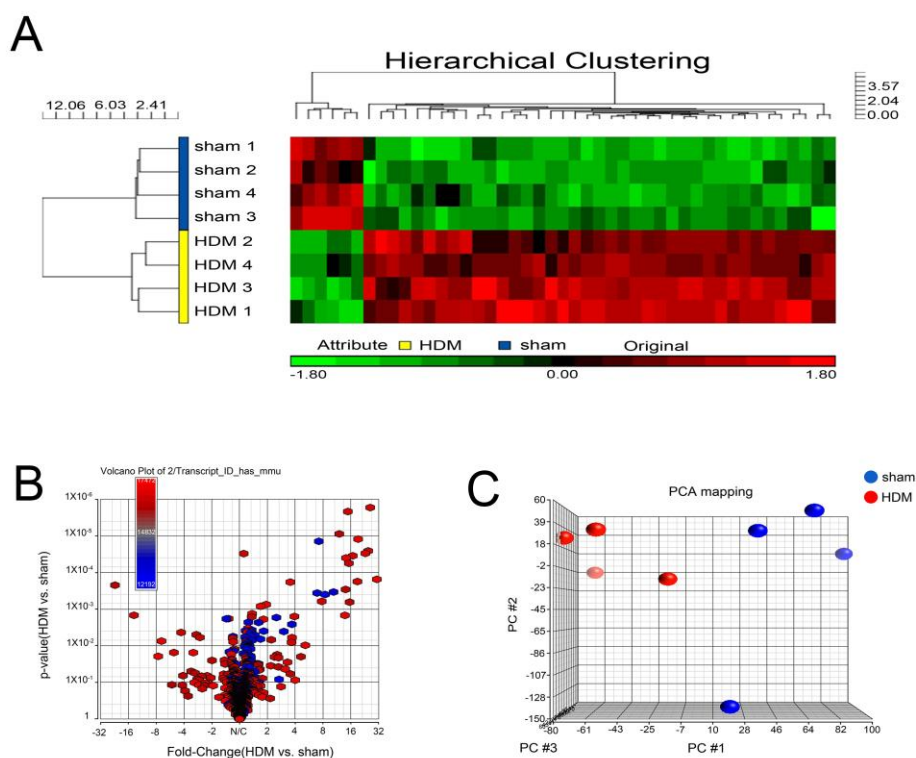


Figure 1



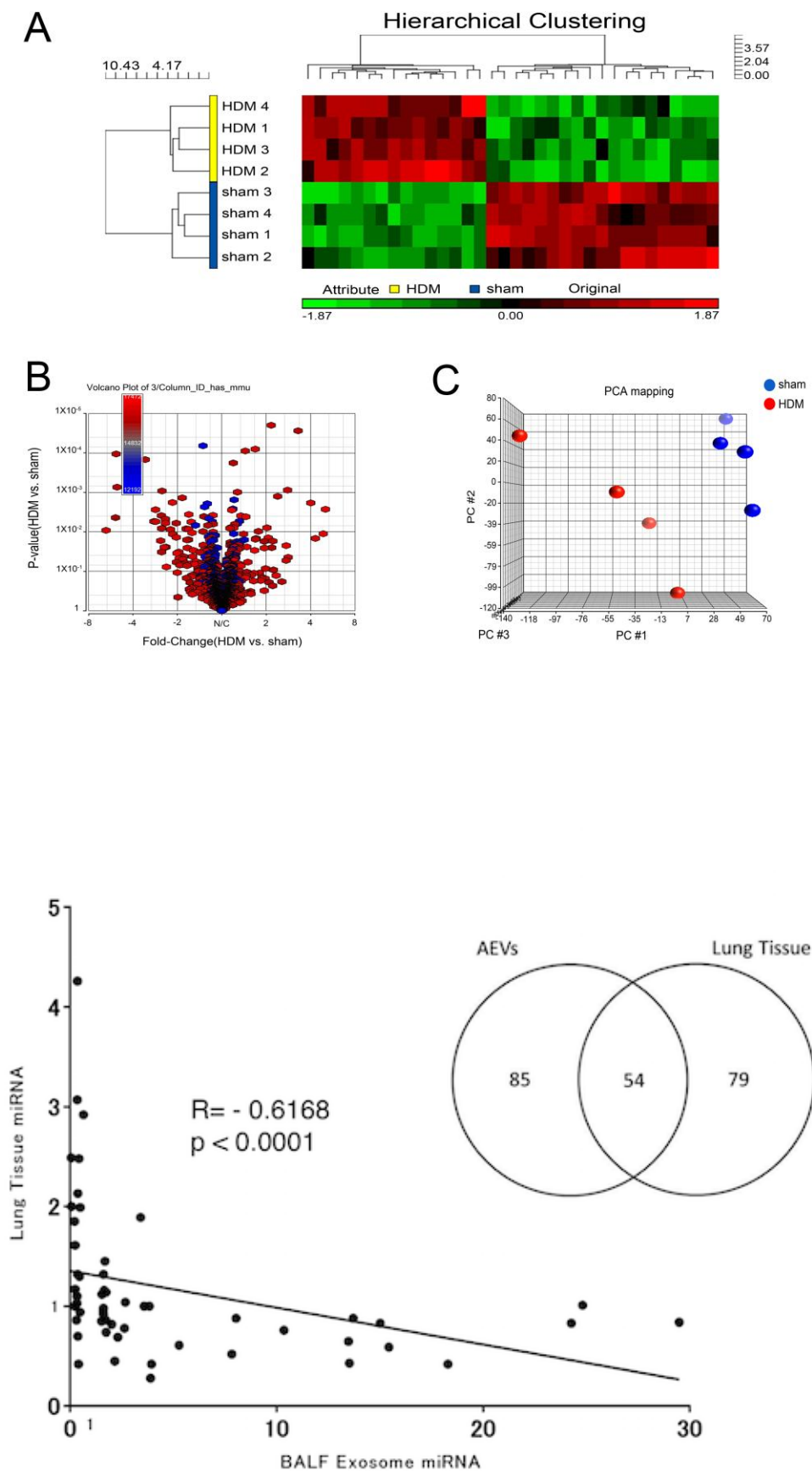


Figure 4

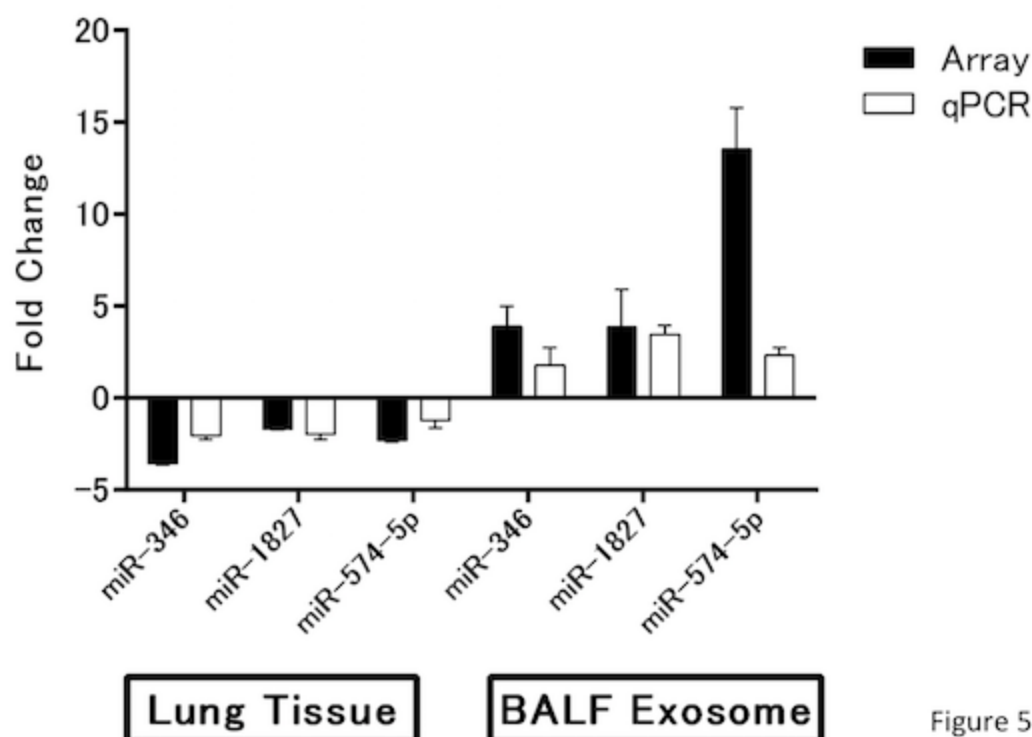


Figure 5

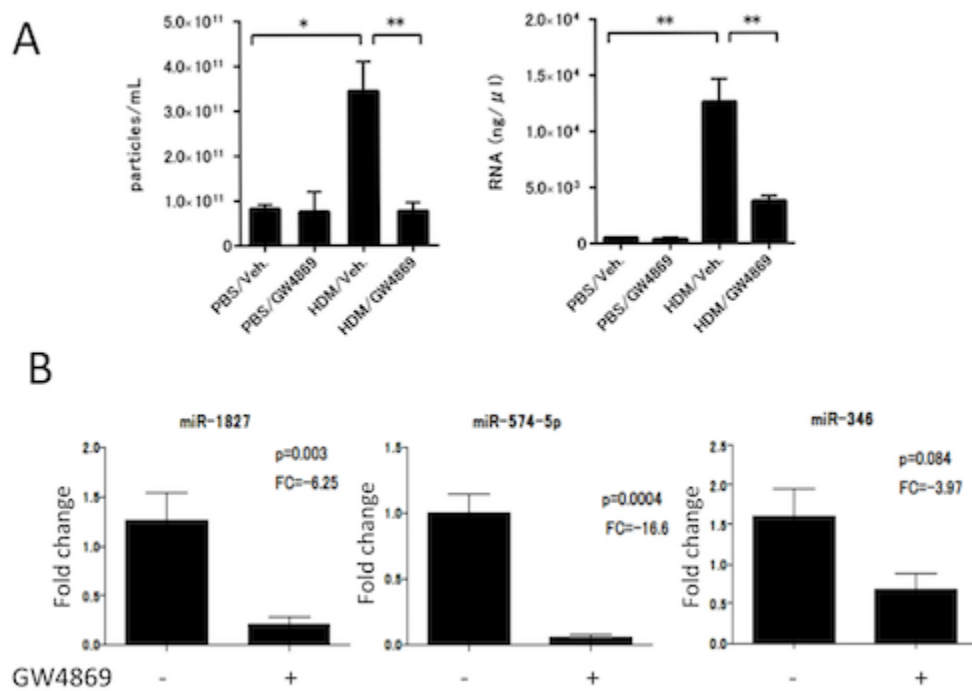


Figure 6

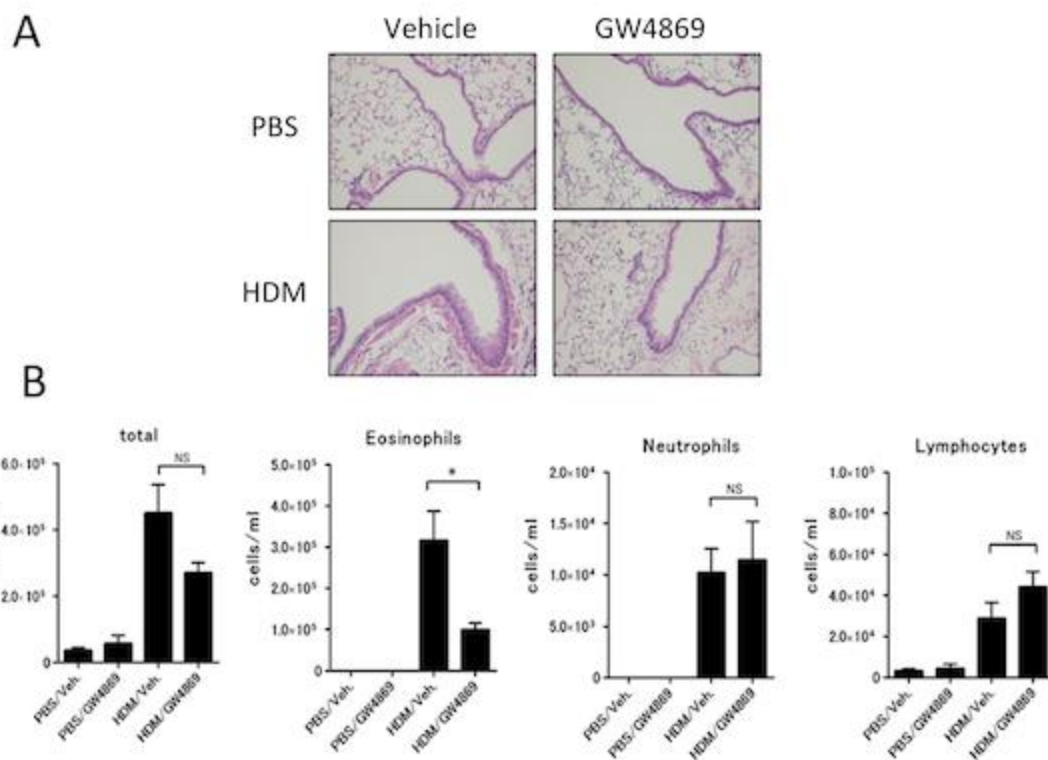


Figure 7

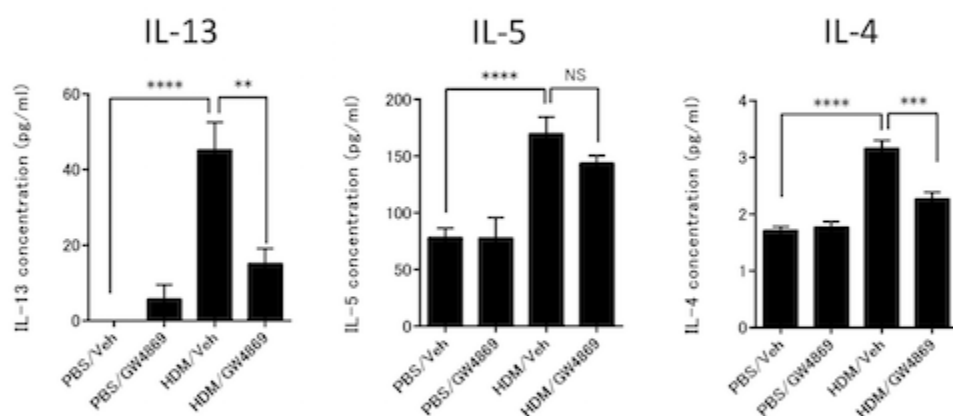
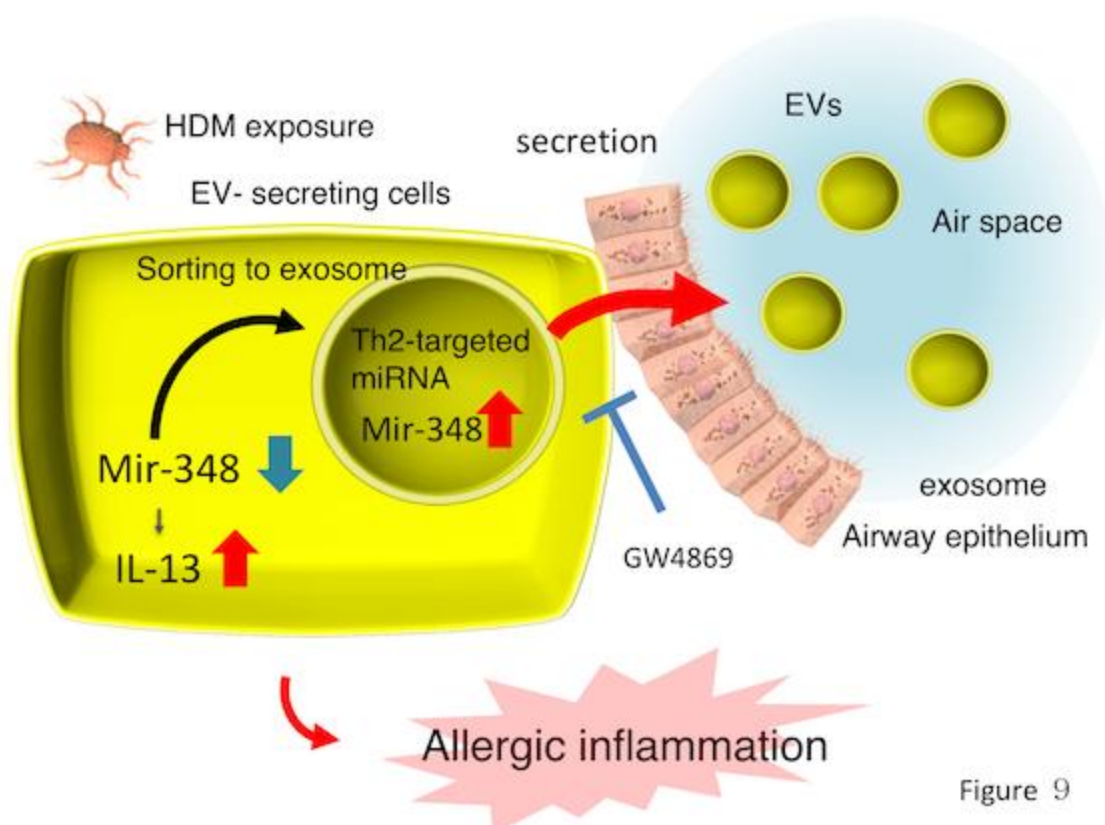


Figure 8



HDM (100 μ g/50 μ l)



D1

HDM (100 μ g/50 μ l)



D7

HDM (100 μ g/50 μ l)



D14

D17

Hierarchical Clustering

