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Human airway epithelial extracellular vesicle miRNA signature is altered upon asthma development

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Abstract

Background: miRNAs are master regulators of signaling pathways critically involved in asthma and are transferred between cells in extracellular vesicles (EV). We aimed to investigate whether the miRNA content of EV secreted by primary normal human bronchial epithelial cells (NHBE) is altered upon asthma development.

Methods: NHBE cells were cultured at air-liquid interface and treated with interleukin (IL)-13 to induce an asthma-like phenotype. EV isolations by precipitation from basal culture medium or apical surface wash were characterized by nanoparticle tracking analysis, transmission electron microscopy, and Western blot, and EV-associated miRNAs were identified by a RT-qPCR-based profiling. Significant candidates were confirmed in EVs isolated by size-exclusion chromatography from nasal lavages of children with mild-to-moderate (n = 8) or severe asthma (n = 9), and healthy controls (n = 9).

Results: NHBE cells secrete EVs to the apical and basal side. 47 miRNAs were expressed in EVs and 16 thereof were significantly altered in basal EV upon IL-13 treatment. Expression of miRNAs could be confirmed in EVs from human nasal lavages. Of note, levels of miR-92b, miR-210, and miR-34a significantly correlated with lung function parameters in children ($FEV_1FVC_{\%pred}$ and $FEF_{25-75\%pred}$), thus lower sEV-miRNA levels in nasal lavages associated with airway obstruction. Subsequent ingenuity pathway analysis predicted the miRNAs to regulate Th2 polarization and dendritic cell maturation.

Conclusion: Our data indicate that secretion of miRNAs in EVs from the airway epithelium, in particular miR-34a, miR-92b, and miR-210, might be involved in the early development of a Th2 response in the airways and asthma.

KEYWORDS
airway epithelium, asthma, extracellular vesicles, miRNA, Th2 polarization
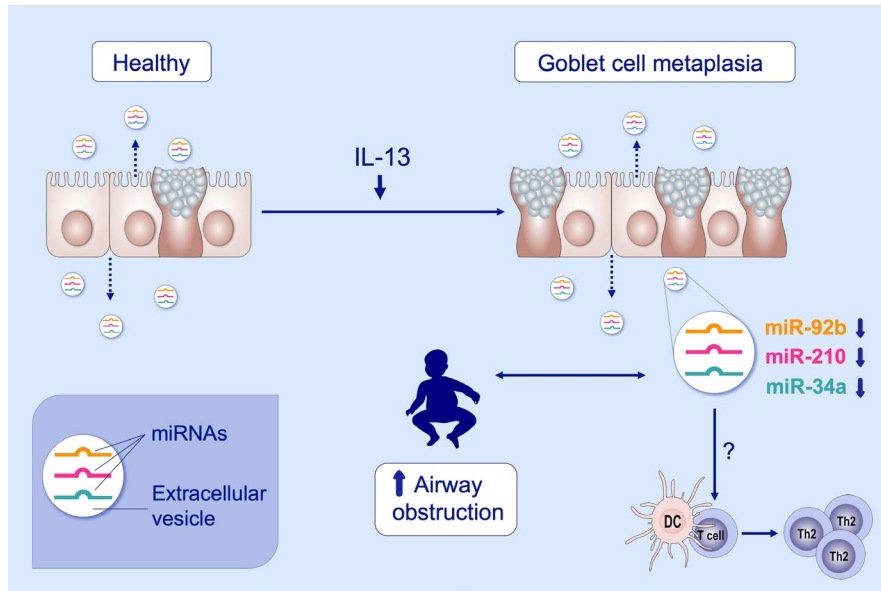
GRAPHICAL ABSTRACT

Primary human bronchial epithelial cells secrete microRNA-containing extracellular vesicles (EV) apically and basally. Upon Interleukin-13-induced goblet cell metaplasia, miR-34a, miR-92b, and miR-210 levels in EVs are lower, which is predicted to influence dendritic cell maturation and Th2 polarization. Lower levels of these microRNAs in EVs in nasal lavages correlate with increased airway obstruction in children.

1 | INTRODUCTION

Worldwide, the incidence and prevalence of chronic inflammatory diseases including asthma are rapidly increasing, and the World Health Organization estimates 300 million people to be suffering from asthma.[1] Thus, there is a huge and unmet need to develop novel therapies and preventative strategies. An important prerequisite for this is a detailed understanding of underlying molecular mechanisms.

The airway epithelium forms a first barrier between inhaled substances and the lung, allowing transmitting environmental signals to the immune system which then mounts an appropriate response. This is crucial for maintaining the balance between defense against inhaled pathogens and tolerance toward innocuous allergens. Environmental stressors, such as virus infections or protease-containing allergens, may influence this system leading to the development of allergies, asthma, or other chronic lung diseases.[2] Interleukin (IL)-33 and other pro-inflammatory alarmins are released upon epithelial damage inducing a Th2 polarization by dendritic cells (DC).[3] However, it is not yet clear if this is alone sufficient to induce the strong and persistent (DC-mediated) Th2 immune response that is characteristic for allergic asthma. Thus, the epithelial-immune cross talk in the lung microenvironment may additionally involve other players, such as extracellular vesicles (EVs).

EVs are subdivided in different classes according to size and intracellular origin, accordingly small EVs (sEVs) (50-150 nm) and derived from multivesicular bodies of late endosomes are typically called exosomes.[4] EVs can transfer microRNAs (miRNA) and express immune-relevant surface molecules such as HLA-DR or co-stimulatory molecules, making them central players in the immune response.[5]

The EV-associated miRNA content changes dynamically upon cellular stress or stimulation.[6] and as miRNAs are master regulators of gene expression[7] this can profoundly affect recipient cells. Several miRNAs are critically involved in asthma,[8-12] and levels within bronchoalveolar lavage fluid (BALF) of patients with asthma are different compared to healthy controls.[13]

Thus, we here aimed to elucidate if the EV-associated miRNA secretion of the airway epithelium is involved in early asthma development. Therefore, we characterized the miRNA secretion in sEVs by primary normal bronchial epithelial cells (NHBE) treated with IL-13 to induce an asthma-like epithelium, which was analysed by ingenuity pathway analysis (IPA) and confirmed in sEVs from human nasal lavages of children with asthma or healthy controls.

2 | MATERIAL AND METHODS

A detailed description of the cell culture, RT-qPCR, Western blot and flow cytometry can be found in this article's online supplement.

2.1 | Air-liquid interface (ALI) culture of NHBE cells

Primary NHBE cells from three healthy individuals were purchased from Lonza (Basel, Switzerland) and cultured as previously described.[9] ALI culture was performed on Collagen IV-coated (Sigma Aldrich, St. Louis, USA) transwell inserts (Polyester, 0.4 μm pore size) (Costar, Washington DC, USA) for 28 days, with Pneumacult-ALI (STEMCELL Technologies, Vancouver, Canada) containing 100 U/ml penicillin and 0.1 mg/mL streptomycin (PAN Biotech, Aidenbach,

Germany) and recombinant human IL-13 in treated wells (5 ng/mL) (R&D systems, Minneapolis, USA).

2.2 | Histology of cross-sectional membranes

After washing the surface with Hank's Balanced Salt Solution (HBSS), cells were fixed by 4% paraformaldehyde (PFA) for 30 minutes at RT. Membranes were stabilized in 2% agarose (Sigma Aldrich), dehydrated, and paraffin-embedded. 1.5 μm membrane cross sections was stained with Periodic acid-Schiff (PAS) staining, indicating mucus in pink, as previously described.[14]

2.3 | RNA Isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total miRNA-enriched RNA was isolated from cells (miRNeasy Mini Kit, Qiagen, Venlo, the Netherlands) and EVs (miRNeasy Micro Kit) according to the manufacturer's recommendations. mRNA was detected by SYBR green based RT-qPCR (Roche, Mannheim, Germany) with specific primers (Table S1) on a Light Cycler 480II platform (Roche). For miRNA PCRs, 10 μL of each EV RNA sample was transcribed using the miScript RT-qPCR system (Qiagen). Differences in expression were calculated by the $\Delta\Delta Ct$ method[15] relative to a mean of the reference genes *HPRT* and *YWHAZ* or a miRNA reference (here miR-25 as it was found equally expressed). Graphs indicate dCp values (dCp = $(Cp_{target} - Cp_{reference\ gene})$, lower value indicates higher expression, thus displayed on reversed *y*-axis).

2.4 | EV precipitation

1 mL of basal medium or apical surface wash (500 μL of HBSS was added per 12-well insert and then re-collected) was incubated overnight at 4°C with 1/4th volume (250 μL) of ExoQuick-TC reagent (System Biosciences, Paolo Alto, USA). EVs were pelleted the next day by centrifuging at 1500 g for 30 minutes.

2.5 | Nanoparticle tracking analysis

EVs were applied to a PMX 110 Scanning ZetaView (Particle Metrix, Meerbusch, Germany) in an appropriate dilution. Two video cycles were recorded at constant temperature and conductivity, each scanning 11 distinct positions in the cell cross section (30 frames/s). Recommended parameters (minimum brightness: 20, shutter: 70, sensitivity: 85, min size: 5 nm, max size: 1000 nm) were chosen, and results were analysed by ZetaView 8.04.02 SP2. Due to low EV counts, we did not include a threshold for completed traces. For NHBE experiments, a median of 47.50 ± 106.1 traces and for nasal lavages a median of 383.0 ± 598.8 traces was measured.

2.6 | Transmission electron microscopy

EVs were fixed in 2% PFA and bound to formvar/carbon-coated 200-mesh nickel grids (Electron Microscopy Sciences, Hatfield,

USA) for 15 minutes. Subsequently, grids were washed in PBS, fixed in 2.5% glutaraldehyde for 5 minutes and washed again in filtered water. EVs were negatively stained with 2% uranyl acetate for 1 minutes, washed, and air-dried overnight. Electron microscopy was performed on a Zeiss EM900 (Carl Zeiss Microscopy GmbH, Jena, Germany) with a wide-angle dual-speed 2K-CCD camera at 80 kV.

2.7 | Western blot

EV proteins were isolated from 10 mL of pooled medium or apical washes according to[16] by using 10× RIPA buffer (Merck Millipore, Burlington, USA) with protease inhibitors (complete Mini, Roche, Mannheim, Germany). We used the Bio-Rad V3 Western blot workflow (Bio-Rad, Hercules, USA) including stain-free technology and the primary antibodies anti-CD63 (EPR5702) (Abcam, Cambridge, UK), anti-CD9 (D3H4P), and anti-Alix (E4T7U) (all rabbit, all Cell Signalling Technology. Danvers. USA) and secondary HRP-conjugated goat anti-rabbit IgG antibody. Chemiluminescence signal was compared to total protein content by using ImageLab V5.2.1 (Bio-Rad).

2.8 | SeramiR miRNA profiling

EV RNA was profiled with the Human SeramiR Exosome RNA Profiling Kit (Systems Biosciences) according to the manufacturer's recommendations. Due to low RNA content of EV samples hampering reliable quantification, we used 10 μL of each sample for reverse transcription. Profiling PCRs were conducted in 96-well plates on a Light Cycler 480 platform (Roche) using specific primers for 384 different human miRNAs.

2.9 | Statistical analysis of miRNA profiling

Profiling data were analysed by the $\Delta\Delta Ct$ method and normalized to included spike-in RNAs. Subsequently, we performed unpaired t tests with Benjamini-Hochberg correction to adjust for multiple testing with the R package limma. An adjusted *P*-value or false discovery rate (FDR) of ≤ 0.1 was set as cut-off for subsequent analysis.

2.10 | Ingenuity pathway analysis

miRNA profiles were analysed by ingenuity pathway analysis (IPA, version 46 901 286), using the features *core analysis* and *miRNA target filter*. All miRNAs regulated with a FDR ≤ 0.1 were included. miRNA target filters were set to *dendritic cell maturation*, *Th1 response* and *Th2 response*, and only *experimentally observed* or *prediction with high confidence* targets were considered.

2.11 | Isolation of small EVs by size-exclusion chromatography

qEV original columns (Izon Biosciences, Oxford, UK) were used according to the manufacturer's instructions. 10 mL of basal medium or

TABLE 1 Clinical and population characteristics

Parameter	Healthy controls, median, IQR	Mild-to-moderate asthma, median, IQR	Severe asthma, median, IQR
No.	9	8	10
Population characteristics			
Female sex, no. (%)	5 (55.6)	3 (37.5)	1 (10.1)
Ethnicity	Caucasian	Caucasian	Caucasian
Age (y)	11.00 (7.50-12.00)	11.00 (9.00-13.00)	1 0.50 (09.00-12.00)
BMI (kg/m^2)	18.96 (17.65-20.19)	18.28 (16.58-21.98)	18.34 (15.61-22.63)
Smoking at home, no. (%)	1 (11.11)	5 (62.50)	3 (30.00)
Clinical characteristics			
FEV$_{1\%pred}$ (%)	110.9 (78.60-118.4)	83.60 (80.53-102.4)	83.10 (77.88-89.45)
FEV$_1$/FVC$_{\%pred}$ (%)	103.7 (91.69-107.00)	94.43 (91.33-102.5)	97.80 (92.98-105.20)
FEF$_{25-75\%pred}$ (%)	99.94 (78.88-108.10)	84.49 (64.28-91.84)	81.75 (65.86-92.46)
Serum IgE [U/ml]	0.00 (0-0)	326.0 (101.40-421.00)	131.60 (42.29-1009)
Blood Eosinophils (%)	0.00 (0-0)	0.58 (0.44-6.60)	1 (0.65 - 17.65)
Oral FeNO (ppb)	0.00 (0-0)	19.5 (7.5-37.75)	9 (5.75-11.75)$^\$$
Nasal nNO (ppb)	0.00 (0-0)	202 (93.00-2007.00)	2050 (347.00-2057.00)
Skin prick test to Dermatophagoides pt, SPT+ (-)	0 (9)	8 (0)	7 (3)
Medication (none, ICS, ICSLABA)	9—0—0	0—2—6	0—0—10

Note: No significant differences between mild-to-moderate/severe asthma vs healthy controls (Mann-Whitney U test, *P* < .05)

Abbreviations: n.a., not assessed.

$^\$$Mann-Whitney U test *P* < .05 vs mild-to-moderate asthma

apical surface wash from pools of cultures (or 3 mL of human nasal lavage) were preconcentrated by 10 kDa Amicon Ultra-4 Filter Units (Merck Milipore) centrifuging at 4000 g for 20 minutes. The remaining suspension was filtered through a 0.2 μm filter (Merck Milipore), adjusted to 500 μL and applied on qEV columns. We collected fractions 1-6 (3 mL), 7-9 (1.5 mL), and 10-15 (3 mL) in separate tubes. Fraction 7-9 (EV-containing) was again concentrated via Amicon filters for further use.

2.12 | Clinical subjects

Children with mild-to-moderate or severe asthma and healthy controls were recruited at the Pediatric Allergology & Pulmonology outpatient clinic of IBIM CNR, Palermo, Italy. Included children had a mean age of 11 years and were selected based on doctor-diagnosed asthma present for at least one year, according to ERS/ATS guidelines (Table 1). 12 age-matched subjects without history of asthma, wheezing, or allergy (skin prick test negative) were assessed as healthy controls.

2.13 | Nasal lavages

6 mL of sterile saline fluid was injected into the nostrils, and the flow-through was collected in a separate tube. Cell-free supernatant was obtained by centrifuging for 10 minutes at 300 g and 4°C and was frozen at −80°C until further use.

2.14 | Statistical analysis

All statistical analysis was performed in GraphPad Prism 6. We used unpaired t test with Welsh's correction for analyzing in vitro experiments and Mann-Whitney U test for clinical data. A *P*-value ≤ .05 was considered statistically significant. Correlations of two parameters were analysed by Spearman correlation analysis. For multiple comparisons, *P*-values were adjusted by Bonferroni correction for the number of comparisons.

3 | RESULTS

3.1 | Primary NHBE cells secrete small EVs to the apical and basal side

To investigate the sEV-miRNA secretion of the airway epithelium, we cultured primary NHBE cells at the air-liquid interface (ALI) (Figure 1A). After 28 days, the differentiated epithelium was pseudostratified containing both ciliated and secretory cell types (Figure 1B). Treatment with IL-13 from d14-d28 increased goblet cells and mucus, confirmed by histology of membrane cross sections (Figure 1B) and increased gene expression of *Mucin5AC* (*MUC5AC*) and *Calcium-activated chloride channel* (*CLCA1*) (Figure 1C). In contrast, the markers *Forkhead Box J1* (*FOXJ1*) for ciliated cells and *Clara Cell 10kDa protein* (*CC10*) for club cells decreased by IL-13 treatment (Figure 1C). Early IL-13 treatment (d0-d14) followed by normal medium for the remaining 14 days,
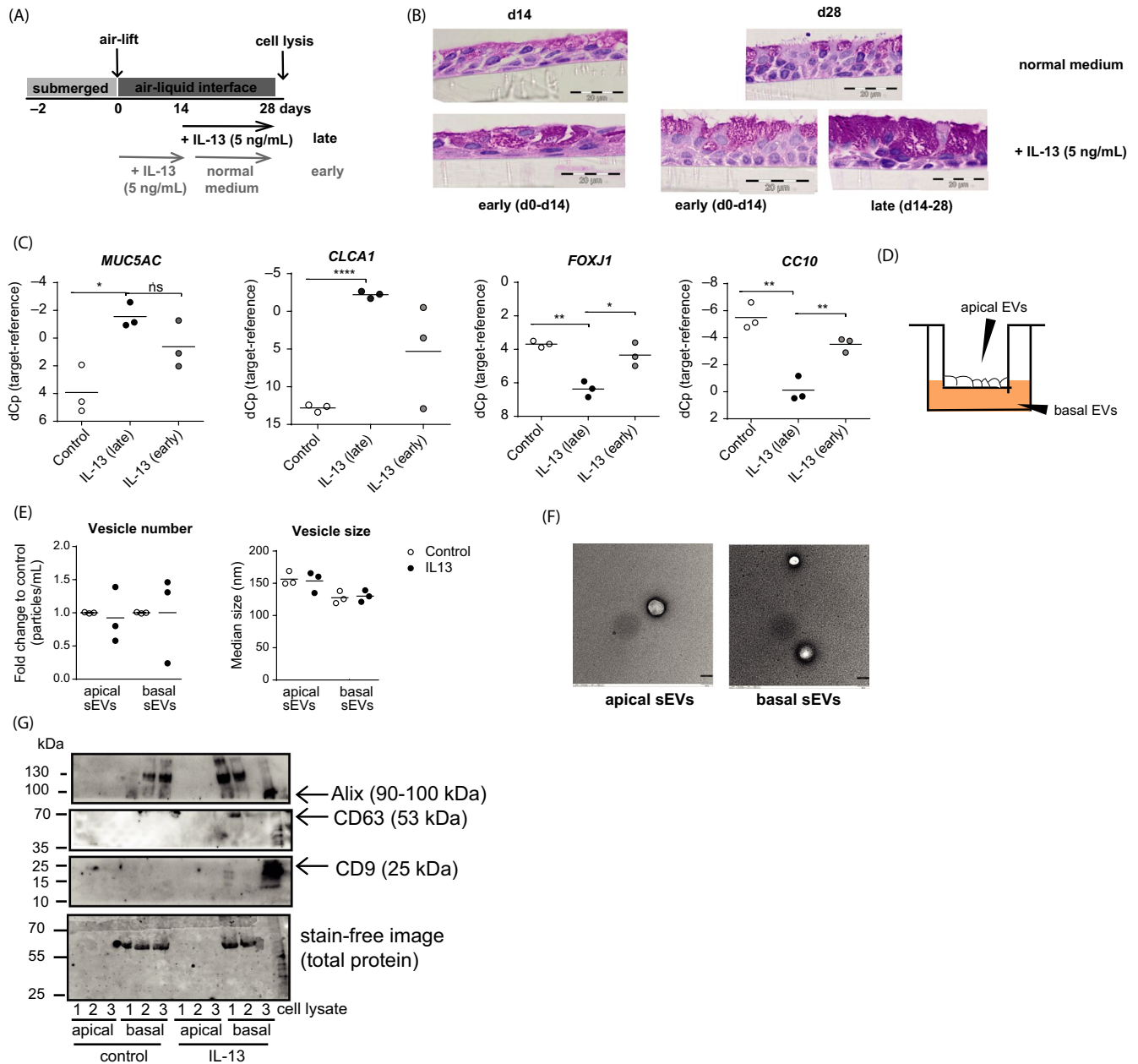
FIGURE 1 Primary bronchial epithelial cells secrete small extracellular vesicles (EVs) to the apical and basal side. A, Scheme of the air-liquid interface differentiation. B, PAS staining of membrane cross sections (representative images of 3 different donors). C, RT-qPCR for cell-type specific genes; n = 3 different experiments with 3 different NHBE donors; dCp = (Cp_{target} − $Cp_{reference\ gene}$), lower value indicates higher expression, thus displayed on reversed y-axis in graph). ****P < .001, **P < .01, *P < .05, unpaired t test with Welch's correction. D, sEVs were isolated from apical surface wash or basal culture medium by precipitation. E, Vesicle concentration (left panel) and vesicle size (right panel) assessed by nanoparticle tracking analysis. F, Transmission electron microscopy (representative images shown of n = 3 isolation of 3 different donors). G, Western blot for Alix, CD63, and CD9 (showing n = 3 different donors) and corresponding stain-free image showing total protein content

allowed cells to partly regenerate this phenotype (Figure 1A-C). As NHBE cells have a distinct apical to basal polarity, we isolated EVs from apical surface washes and basal culture medium by precipitation (Figure 1D). Nanoparticle tracking analysis (NTA) (Figure 1E) and transmission electron microscopy (TEM) (Figure 1F) confirmed the presence of small (<200 nm) EVs both apically and basally. Western blot analysis revealed that basal sEVs were positive for the EV-marker Alix and in part CD63 (Figure 1G), while markers on apical sEVs could

not be detected via Western blot due to insufficient protein levels (Figure 1-E-G).

3.2 | IL-13 treatment alters the miRNA content of epithelial sEVs

Next, we profiled the expression of 384 miRNAs each in apical and basal sEVs from control, early IL-13- and late IL-13-treated cells. In
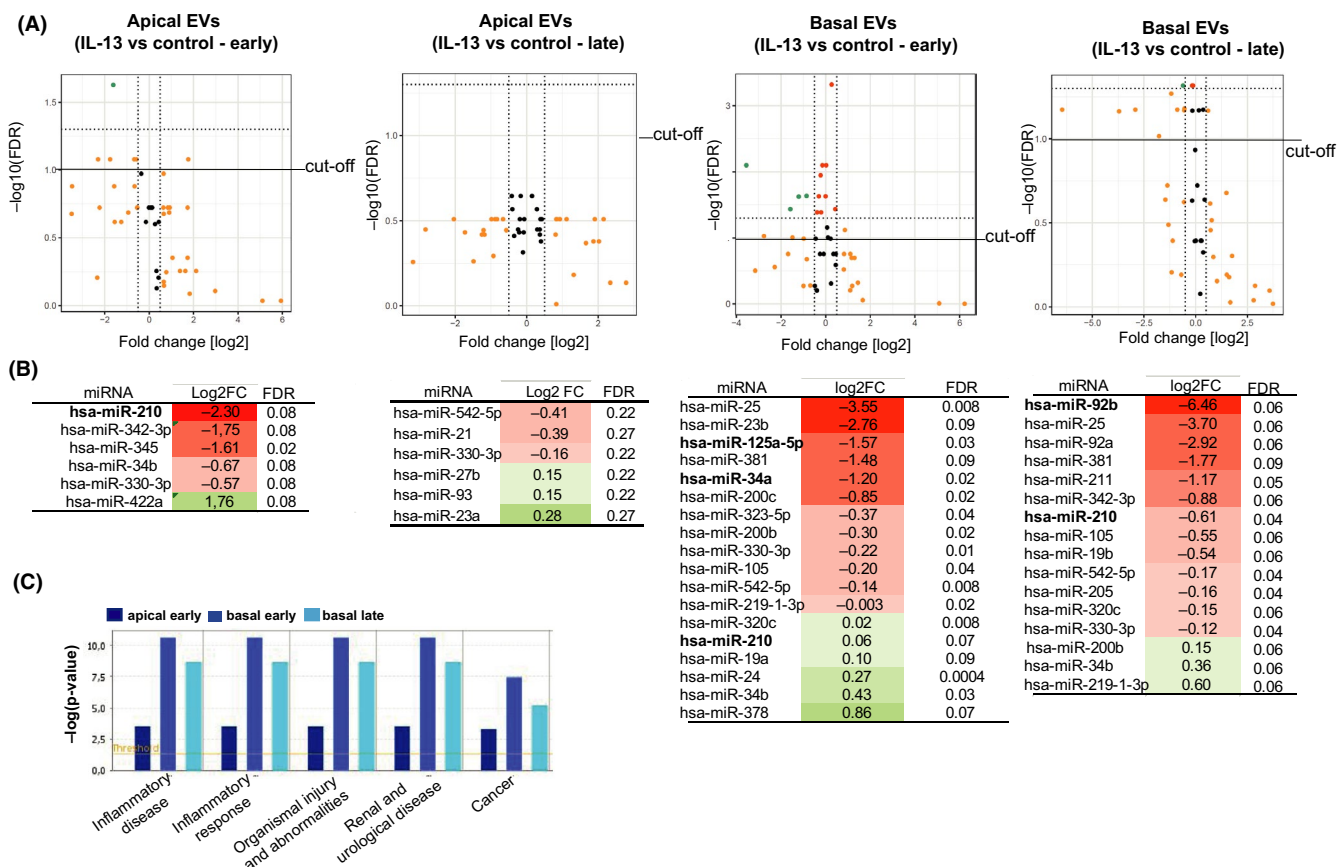
(A)

(B)

Apical EVs (IL-13 vs control – early)

miRNA	Log2FC	FDR
hsa-miR-210	−2.30	0.08
hsa-miR-342-3p	−1.75	0.08
hsa-miR-345	−1.61	0.02
hsa-miR-34b	−0.67	0.08
hsa-miR-330-3p	−0.57	0.08
hsa-miR-422a	1.76	0.08

Apical EVs (IL-13 vs control – late)

miRNA	Log2 FC	FDR
hsa-miR-542-5p	−0.41	0.22
hsa-miR-21	−0.39	0.27
hsa-miR-330-3p	−0.16	0.22
hsa-miR-27b	0.15	0.22
hsa-miR-93	0.15	0.22
hsa-miR-23a	0.28	0.27

Basal EVs (IL-13 vs control – early)

miRNA	log2FC	FDR
hsa-miR-25	−3.55	0.008
hsa-miR-23b	−2.76	0.09
hsa-miR-125a-5p	−1.57	0.03
hsa-miR-381	−1.48	0.09
hsa-miR-34a	−1.20	0.02
hsa-miR-200c	−0.85	0.02
hsa-miR-323-5p	−0.37	0.04
hsa-miR-200b	−0.30	0.02
hsa-miR-330-3p	−0.22	0.01
hsa-miR-105	−0.20	0.04
hsa-miR-542-5p	−0.14	0.008
hsa-miR-219-1-3p	−0.003	0.02
hsa-miR-320c	0.02	0.008
hsa-miR-210	0.06	0.07
hsa-miR-19a	0.10	0.09
hsa-miR-24	0.27	0.0004
hsa-miR-34b	0.43	0.03
hsa-miR-378	0.86	0.07

Basal EVs (IL-13 vs control – late)

miRNA	log2FC	FDR
hsa-miR-92b	−6.46	0.06
hsa-miR-25	−3.70	0.06
hsa-miR-92a	−2.92	0.06
hsa-miR-381	−1.77	0.09
hsa-miR-211	−1.17	0.05
hsa-miR-342-3p	−0.88	0.06
hsa-miR-210	−0.61	0.04
hsa-miR-105	−0.55	0.06
hsa-miR-19b	−0.54	0.06
hsa-miR-542-5p	−0.17	0.04
hsa-miR-205	−0.16	0.04
hsa-miR-320c	−0.15	0.06
hsa-miR-330-3p	−0.12	0.04
hsa-miR-200b	0.15	0.06
hsa-miR-34b	0.36	0.06
hsa-miR-219-1-3p	0.60	0.06

(C)

FIGURE 2 IL-13 treatment alters the miRNA content of epithelial EVs. A, Plots showing significantly changed miRNAs in basal EVs and apical EVs upon early (d0-d14) or late (d14-d28) IL-13 treatment of NHBE cells (cut-off FDR = 0.1). B, Corresponding heatmaps of IL-13 regulated miRNAs in EVs. Red indicates downregulation upon IL-13 treatment vs control, green indicates upregulation. Selected candidate miRNAs are indicated in bold. C, Prediction of effect of altered sEV-miRNA content by ingenuity pathway analysis (IPA)

total, 47 miRNAs were expressed in EVs with a Cp ≤ 35. In apical sEVs of early IL-13-treated cells six miRNAs were significantly altered compared to control cells, while there was none that reached statistical significance (in apical EVs) upon late IL-13 treatment (cut-off false detection rate (FDR)<0.1) (Figure 2A). In basal EVs, 18 miRNAs were significantly altered in basal EVs upon early IL-13 treatment and 16 due to late IL-13 treatment (Figure 2 A, B, Table S2). Notably, the majority of miRNAs were downregulated, with miR-92b showing the strongest regulation (logFC −6.46 in basal EVs after late IL-13 treatment, FDR = 0.06) (Figure 2B). Ingenuity pathway analysis (IPA) identified that IL-13-induced alterations of miRNA levels in both apical and basal sEVs were most significantly associated with inflammatory disease (P-value range of: basal sEVs (d0-d14) = 3.11e-02-2.27e-11; basal sEVs (d14-d28) = 2.88e-02-2.14e-09; apical sEVs (d0-d14) = 4.90e-02-3.00e-04) and inflammatory response (Table S3), while the prediction was more significant for basal profiles (Figure 2C, Table S3).

3.3 | Confirmation of changes in sEVs isolated by size-exclusion chromatography (SEC)

Precipitation-based EV isolation co-precipitates free protein-bound miRNA that are not enclosed in EVs.[17] To confirm that

significantly regulated miRNAs are truly EV-associated and can therefore be transferred within the lung microenvironment, we isolated sEVs by SEC, having a superior separation from residual protein.[18] SEC sEVs were < 200 nm, express CD63, CD9, and CD81 (Figure S1C, S2), but both particle concentrations and marker expression did not change upon IL-13 treatment (Figure S1 A-C, S2). In line with precipitated sEVs, IL-13 induced a strong significant decrease of miR-125a-5p in apical sEVs ($FC_{miR-125a-5p}$ = 0.28; P = .011) and of miR-92b in both apical ($FC_{miR-92b}$ = 0.07; P = .0001) and basal sEVs ($FC_{miR-92b}$ = 0.52, P = .045) (Figure S1 C). miR-210 and miR-34a levels were decreased in both miRNAs in apical sEVs in two of the three NHBE donors (not statistically significant). miR-542-5p and miR-345 (trendwise) were decreased in basal sEVs ($FC_{miR-345}$ = 0.52; P = .06, $FC_{miR-542-5p}$ = 0.228; P = .009) after IL-13 treatment (Figure S1 C).

3.4 | sEV-miRNA levels in nasal lavages correlate with airway obstruction in children

To translate our findings to the human situation, we assessed the miRNA levels in sEVs from nasal lavages of children with mild-to-moderate (AM) or severe asthma (AS) compared to healthy controls
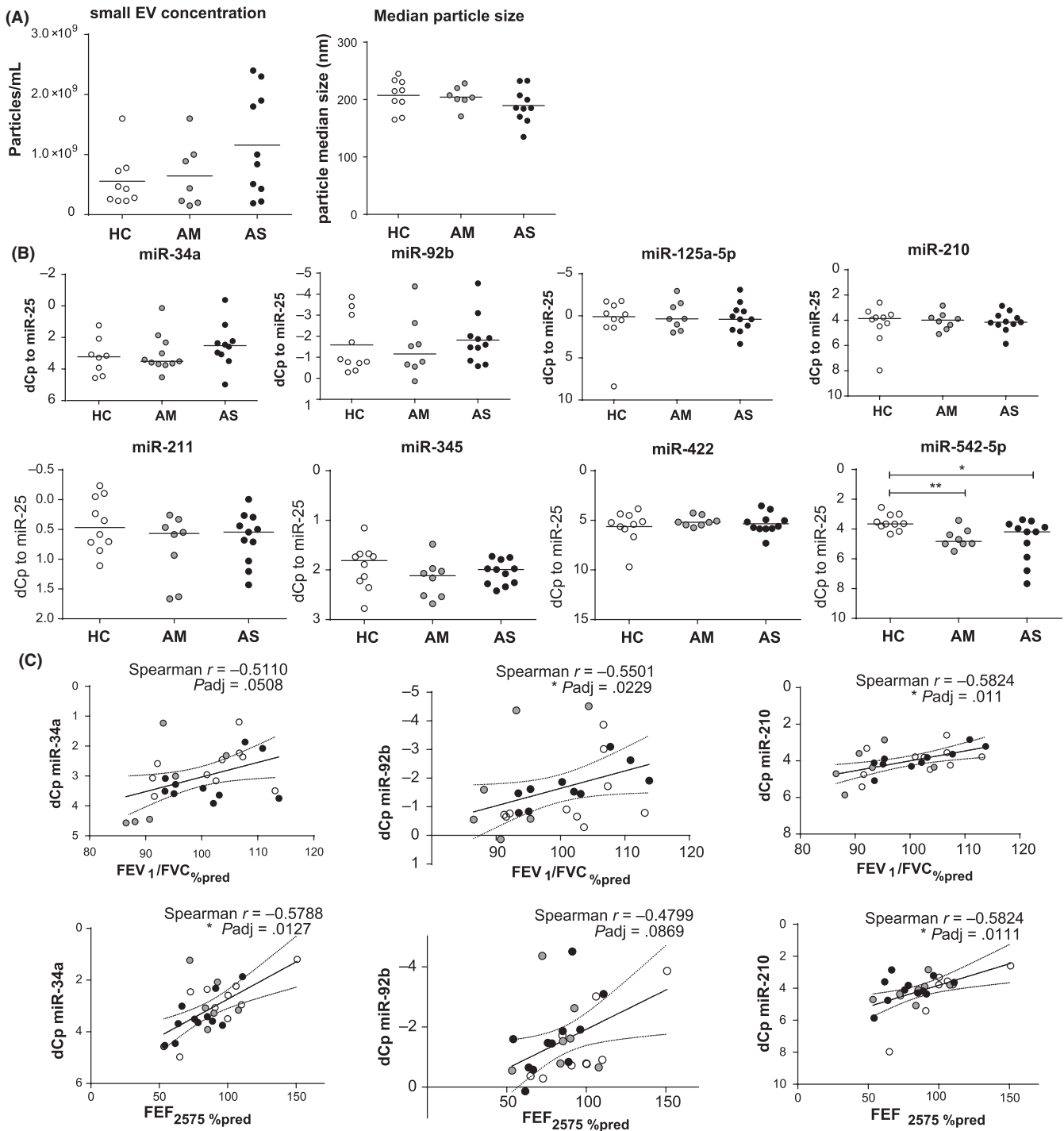
FIGURE 3 Small sEV-miRNA levels in nasal washes correlate with airway obstruction in children. A, Vesicle concentration (left panel) and vesicle size (right panel) assessed by nanoparticle tracking analysis from nasal wash of healthy control children (HC) (n = 9), and children with mild-to-moderate asthma (AM) (n = 8) or severe asthma (AS) (n = 10); Mann-Whitney U test vs HC. B RT-qPCR of candidate miRNAs of isolated sEVs plotted as dCp values (dCp = (Cp_{target} − $Cp_{reference\ miRNA}$, lower value indicates higher expression, thus displayed on reversed y-axis in graph). Mann-Whitney U test vs HC. C Spearman correlations of miRNA levels (plotted as dCp) in sEVs from nasal washes with $FEV_1FVC_{\%pred}$ (upper panel) and $FEF_{25-75\%pred}$ (lower panel); Spearman r value and adjusted P values indicating significance of correlation are shown in the respective graphs. White dots represent healthy controls, gray dots mild-to-moderate asthma, and black dots severe asthma. Lines indicate linear regression with confidence intervals

(HC). Concentration and size of SEC-isolated EVs from nasal lavage did not differ among the patient groups (Figure 3A). All previously identified miRNAs were expressed in nasal lavage sEVs, while

miR-542-5p showed a significant decrease in AM and AS groups (Figure 3B). miR-92b (Spearman r = −.55, P_{adj} = .02) and miR-210 (Spearman r = −.58, P_{adj} = .01) levels significantly correlated with

TABLE 2 Correlation of sEV-miRNA levels with clinical characteristics

Parameter	FEV$_1$FVC$_{\%pred}$	FEV$_{1\%pred}$	FEF$_{2575\%pred}$	Nasal nNO [ppb]	Serum IgE [U/mL]	Blood eosinophils [%]
dCP EV miR-34a	**−0.511 (0.051)**	−0.245 (0.860)	**−0.579 (0.013)***	−0.472 (0.468)	0.414 (0.660)	0.355 (0.933)
dCP EV miR-92b	**−0.550 (0.023)***	−0.012 (1.000)	−0.480 (0.087)	−0.295 (0.926)	0.568 (0.214)	0.255 (0.992)
dCP EV miR-125a-5p	−0.345 (0.478)	−0.123 (0.998)	−0.400 (0.271)	−0.583 (0.177)	0.532 (0.301)	0.255 (0.992)
dCP EV miR-210	**−0.582 (0.011)***	−0.002 (1.000)	**−0.596 (0.008)****	0.515 (0.345)	−0.250 (0.974)	0.164 (1.000)
dCP EV miR-211	0.035 (1.000)	−0.068 (1.000)	0.199 (0.959)	0.038 (01.000)	−0.029 (1.000)	−0.136 (1.000)
dCP EV miR-345	−0.168 (0.984)	−0.174 (0.979)	−0.268 (0.787)	−0.119 (1.000)	−0.288 (0.9374)	−0.191 (0.999)
dCP EV miR-422	−0.410 (0.241)	0.167 (0.9841)	−0.341 (0.493)	−0.063 (1.000)	−0.014 (1.000)	0.118 (1.000)
dCP EV miR-542-3p	−0.161 (0.988)	−0.312 (0.618)	−0.499 (0.063)	−0.351 (0.827)	−0.334 (0.862)	−0.0775 (1.000)

Note: Table lists spearman *r* correlation co-efficient (adjusted *P*-value). Significant correlations are marked in bold. miRNA levels were assessed in sEVs from nasal washes of n = 9 healthy controls, n = 8 mild-to-moderate asthma and n = 10 severe asthma.
*indicates *P* < 0.05.
**indicates *P* < 0.01.

the lung function parameter FEV$_1$FVC$_{\%pre}$, and we could observe a strong trend for miR-34a (Spearman *r* = −0.51, *P*$_{adj}$ = .051) as well. Of note, the latter miRNA also significantly correlated with a parameter for small airway obstruction[19] FEF$_{25-75\%pred}$ (Spearman *r* = −.58, *P*$_{adj}$ = .01) together with miR-210 (Spearman *r* = −.58, *P*$_{adj}$ = .01) and trendwise miR-92b (Spearman *r* = −0.48, *P*$_{adj}$ = .09) (Figure 3C, Table 2). This indicates that lower miR-34a, miR-92b, and miR-210 levels in nasal lavage sEVs are associated with airway obstruction in children enrolled in this study.

3.5 | Altered miRNA levels in sEVs could influence Th polarization by DCs

As pediatric asthma is predominantly allergic (Th2 dominant), we used IPA to search for targets of the miRNAs influencing the maturation of DCs or the polarization of Th cells. We identified a putative network of miR-34a, miR-92b, and miR-210 and their targets associated with both DC maturation and Th2 polarization (Figure 4). Among others, miR-210 was predicted to target *Runt Related Transcription Factor 3 (RUNX3)*, or *Phosphoinositide-3-Kinase Regulatory Subunit 5 (PIK3R5)*, miR-34a targets *NOTCH1, NOTCH2, Jagged 1 (JAG1)*, and *IKAROS Family Zinc Finger 1 (IKZ-F1)*. The latter is also predicted for miR-92b along with *bone morphogenic protein receptor 2 (BMPR2)*. In theory, as the three miRNAs are decreased in sEVs upon IL-13 treatment or in asthma, all of these factors would presumably increase, potentially enhancing DC maturation and Th2 differentiation (Figure 4).

4 | DISCUSSION

We here report for the first time that primary NHBE cells secrete sEVs apically and basally. sEVs derived by precipitation of IL-13-treated epithelial cells contained decreased levels of miR-92b, miR-34a, and miR-210. A lower expression of miR-34a, miR-92b,
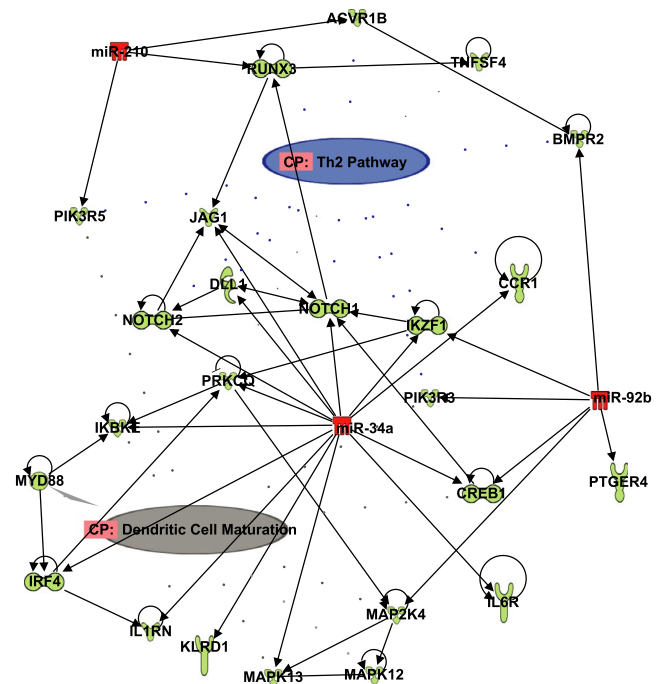

FIGURE 4 miR-92b, miR-34a, and miR-210 are predicted to regulate Th2 differentiation and dendritic cell maturation. IPA-predicted network of miR-92b, miR-34a, and miR-210 showing predicted targets and their association with biological functions *Th2 differentiation* and *dendritic cell maturation*. Red indicates decrease on array, and green indicates predicted increase

and miR-210 levels in sEVs in nasal lavages from children was associated with obstruction of large (FEV$_1$FVC$_{\%pred}$) and small airways (FEF$_{25-75\%pred}$).[19] All three miRNAs were predicted to be involved in regulating Th2 differentiation and DC maturation. In summary, our data suggest that airway epithelial miRNA secretion via sEVs might be implicated in the development of asthma.

Virtually every cell type can produce EVs,[20] including the airway epithelium.[21-23] Here, we isolated sEVs from both apical surface

washes and basal medium of primary NHBE cells and confirmed this by TEM, NTA, and Western blot. We did not further subclassify them into, that is, exosomes, as pure isolations thereof are virtually impossible,[20] and we here were interested to show that the entity of sEVs and their cargo, that also coincide in vivo, is involved in asthma development. Concentration, size, or marker expression did not change upon IL-13 treatment, demonstrating that it specifically affected the miRNA content. The expression of identified miRNAs was confirmed in SEC-isolated sEVs free from contaminating (RNA-binding proteins),[6] indicating that the miRNAs could potentially be transferred between cells.

It is known that sEV-miRNA levels in BALF of patients with asthma differ from healthy controls, and some miRNAs significantly correlated with lung function (FEV$_1$).[24] We can now add that miRNA-containing sEVs can be isolated from nasal lavages, being easier to obtain and less invasive, which is particularly important in pediatric patients. As asthma remains difficult to diagnose in young children, it is intriguing to speculate that sEV-miRNA signatures in nasal lavages could be used accordingly in the future.

The IPA prediction that miR-34a, miR-92b, and miR-210 affect DC function and Th2 polarization is supported by experimental evidence from others: miR-34a is enclosed in sEVs [25] and regulates DC function and maturation via targeting the Wingless/Integrase (Wnt) pathway.[26,27] miR-34a increases in conventional DCs decrease upon antigen encounter and its overexpression suppressed CD4$^+$ T-cell polarization.[27] In rheumatoid arthritis, miR-34a is increased in DCs, and knock-out animals had less severe disease and less IL-17A,[28] again highlighting its important role in DC function. A decrease in miR-34a in airway epithelial sEVs could therewith potentially enhance CD4$^+$ Th2-polarization in asthma.

miR-210 has been found in EVs,[29] is increased in tolerogenic and activated DCs,[30] and further involved in regulatory T-cell function via targeting Forkhead Box 3 (FOXP3).[31] An overexpression of miR-210 resulted in immune dysfunction and less immunosuppressive capacity of Tregs in psoriasis vulgaris,[32] and miR-210 induces Th1 and Th17 differentiation but inhibits Th2 in psoriasis.[33] This is in line with our IPA prediction, that an IL-13-induced decrease of miR-210 in sEVs could increase Th2 polarization by DCs. This miRNA has been shown to be increased in sEVs isolated from nasal mucus of adults with allergic rhinitis compared to healthy controls,[34] suggesting a disease specific regulation of nasal sEV-miRNA levels.

miR-92b has not been described in relation to the immune system or asthma, but regulates epithelial-to-mesenchymal transition (EMT) by targeting SMAD3 in nasopharyngeal carcinoma.[35] Further, it is present in serum exosomes serving as biomarker for acute heart failure.[36] The correlation with airway obstruction in children and its IPA-predicted targets relevant for Th2 polarization such as IKZF1 and BMPR2 strongly suggest a new role for this particular miRNA in the development of asthma. Additionally, airway remodeling and loss of airway integrity in asthma is a type of EMT[37] influencing airway obstruction and hyper reactivity, which might be regulated by epithelial sEVs via miR-92b.

Aside from miRNAs, sEVs contribute to asthma pathogenesis via other mechanisms. EVs from the airway epithelium present allergens on MHCII molecules, inducing tolerance or an active immune response depending on the status of the epithelium.[38-40] BALF sEVs include leukotrienes and increased cytokine release from an airway epithelial cell line,[41] while sEVs released from an IL-13-treated bronchial epithelial cell line acted pro-inflammatory in mice and induced macrophage chemotaxis.[23] Furthermore, EVs isolated from nasal secretions of children with asthma or chronic rhinitis increased immune cell trafficking, due to an altered protein cargo.[42] The actions of airway epithelial sEVs are thus multi-potent and supposedly contribute with an entity of different molecules to asthma pathogenesis.

We would like to acknowledge some limitations of our study. We here only use NHBE cells from healthy individuals treated with IL-13 to induce an "asthma-like" phenotype, as we were interested to study the early molecular changes in the secretions of the airway epithelium, which is not possible when using cells from patients with an established disease. Nonetheless, we were able to confirm the expression of all miRNAs in sEVs isolated from nasal lavages, and three miRNAs significantly correlated with airway obstruction in children. Of note, nasal epithelial cells have been suggested as a less-invasive surrogate for bronchial epithelial cells, due to high similarities in their gene expression levels.[43] Second, the numbers of included patients and NHBE donors are low and findings need to be confirmed in larger studies. Third, we are aware that the Western blot-based characterization is relatively poor due to very low yield of proteins isolated from sEVs from primary cell cultures (especially the apical washes).[6] However, we here followed the standard isolation protocols with which both methods have been shown to isolate intact sEVs expressing relevant markers before[44-46] and we could confirm the presence of CD81, CD63, and CD9 on SEC-isolated sEVs by a more sensitive flow cytometry approach. Fourth, a functional transfer of miRNA-containing sEVs between the airway epithelium and DCs needs to be shown in future studies. Albeit, as EVs contain surface molecules such as HLA-DR,[47] we figured that the cellular interaction might be subject-specific and thus question the use of DCs from different human donors. It has however been shown several times that miRNAs can be functionally transferred to target cells [48-53] and that DC function is modulated by EVs.[54-56] Thus, it is intriguing to speculate that sEVs-associated epithelial miRNAs are involved in regulating the immune responses by underlying DCs. Based on our theoretical IPA-model, decreased levels of miR-92b, miR-210, and miR-34a in epithelial-derived sEVs upon asthma development, would allow DCs to polarize Th2 cells, perpetuating the asthmatic phenotype in the lung microenvironment. While the direct transfer of these miRNAs to DCs (or other target cells) needs experimental confirmation in follow-up studies, our results nonetheless represent a first and encouraging insight into a potential role of epithelial EV-associated miRNAs in the lung microenvironment upon asthma development.

CONFLICTS OF INTEREST

SB has acted as a paid scientific advisor and has received funding for research from Bencard Allergie GmbH, none of which is related to the content of this manuscript.

AUTHORS CONTRIBUTIONS

S.K-E. and SB conceived the manuscript and designed the study. SB performed the experiments and data analysis and had primary responsibility for writing the manuscript. GC, AG, AB, GP, and SG collected and processed the human nasal lavage samples, JK performed the biostatistical analysis of the array, JB helped with setting up the flow cytometry assay. SH, DC, and MWP assisted with EV characterization and quantification. All authors contributed to writing of the manuscript and agree with its final version.

ETHICAL APPROVAL

The study was approved by the local ethics committee (n. 8/2014, AOUP "Paolo Giaccone", Palermo, Italy), and written informed consent was provided by 39 parents of all participants. The approved study was registered on the central registration system ClinicalTrials. gov (ID: NCT02433275).

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