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Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma

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Abstract

Background: Leukotrienes (LTs) are potent pro-inflammatory mediators involved in asthma. Exosomes, nanosized vesicles released from various cells, can stimulate or down-regulate immune responses, depending on the state and nature of the originating cell. We have recently shown an altered exosome profile in bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis, but their role in asthma is unknown. Our aims were to investigate whether exosomes from BALF have LT biosynthetic capacity and to explore phenotypic and functional characteristics of BALF exosomes in asthma.

Methods: Bronchoalveolar lavage fluid exosomes were collected from healthy individuals (n = 13) and patients with mild allergic asthma to birch pollen (n = 12) before and after birch allergen provocation. Exosomes were characterized by flow cytometry and Western blot. Their capacity to induce IL-8 and LT production in the human bronchial epithelial cell (BEC) line 16HB14o- was measured by ELISA and reverse-phase HPLC, respectively.

Results: Compared to BALF exosomes from healthy individuals, BALF exosomes from asthmatics displayed higher levels of exosome-associated markers, such as the tetraspanins CD63 and CD81 and the scavenger receptor CD36. No major differences were observed between BALF exosomes from before and after allergen provocation. Furthermore, we show that BALF exosomes contain enzymes for LT biosynthesis. The effect of exosomes to promote LTC_4 and IL-8 release in BEC was significantly increased for exosomes from asthmatics, and the $CysLT_1$ receptor antagonist Montelukast reduced exosome-induced IL-8 secretion.

Conclusions: Bronchoalveolar lavage fluid exosomes from asthmatic and healthy individuals exhibit distinct phenotypes and functions. BALF exosomes from asthmatics might contribute to subclinical inflammation by increasing cytokine and LTC_4 generation in airway epithelium.

Allergic asthma is a pulmonary inflammatory disease characterized by airway obstruction, inflammation, and the presence of allergen-specific serum IgE. Pollen allergy is one of the most common forms of seasonal respiratory allergic disease in Europe, with a major clinical impact as a result (1).

Leukotrienes (LTs) elicit asthma symptoms, such as bronchoconstriction, edema formation, and mucus hypersecretion (2). LT biosynthesis is initiated by the recruitment of

Abbreviations
AA, arachidonic acid; BALF, bronchoalveolar lavage fluid; BEC, bronchial epithelial cell; $CysLT_1$, cysteinyl leukotriene receptor$_1$; DC, dendritic cells; FLAP, 5-lipoxygenase-activating protein; LO, lipoxygenase; LTs, leukotrienes; LTA_4H, leukotriene A_4 hydrolase; LTC_4S, leukotriene C_4 synthase.

cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase (5-LO) to the nuclear membrane, causing the release of arachidonic acid (AA) by cell activation (3). AA is converted to LTA_4 by 5-LO and further metabolized to the leukocyte chemo-attractant LTB_4 (4) by LTA_4 hydrolase (LTA_4H) or to the bronchoconstrictive LTC_4 (5) by LTC_4 synthase (LTC_4S). Efficient anti-asthma therapies targeting the LT pathway have been developed, thus emphasizing their importance in asthma pathology.

Exosomes are nanovesicles (30–100 nm) produced by numerous cells, including immune cells (6). They form in the endosomal compartment and are released upon fusion with the plasma membrane. In 1996, Raposo and colleagues discovered MHC (Major Histocompatibility Complex)-class II and costimulatory molecules on exosomes (7). Since then, exosomes have proven capable of inducing potent innate and adaptive immune responses *in vivo* (8, 9). Immunosuppressive exosomes have also been reported, for example, from tumor cells (10) and intestinal epithelial cells (11). Production of exosomes *in vivo* is supported by their presence in body fluids, such as plasma (12), urine (13), and bronchoalveolar lavage fluid (BALF) (14, 15). The role of BALF exosomes in allergic reactions is unknown. However, we have shown that B-cell exosomes from allergic individuals can present allergen-derived peptides and induce Th2-like cytokine production (16).

We have recently shown that exosomes from human monocyte-derived dendritic cells and macrophages carry functional LT-producing enzymes (17), suggesting a role of exosomes in transcellular LT biosynthesis. We aimed at exploring the potential ability of BALF exosomes in LT production at steady state and during allergen-induced asthmatic responses. We found phenotypic and functional alterations of BALF exosomes from asthmatics compared to controls. BALF exosomes from patients could increase LT formation and IL-8 release in bronchial epithelial cells (BECs), suggesting a possible engagement in inflammatory reactions of the lung.

Methods

Study subjects

Thirteen healthy individuals and 12 birch pollen–sensitized mild asthmatics participated. Three additional birch pollen asthmatics were included for blocking experiments. All patients had birch pollen–specific IgE (≥ 2 kU/l), while controls were negative (≤ 0.35 kU/l) to Phadiatop (Phadia AB, Uppsala, Sweden). For detailed characterization of subjects, see Tables 1 and 2. Patients were free of asthma exacerbations and pulmonary infections 4 weeks prior to sampling. Their asthma had been stable with occasional medications with inhaled β2-agonist. The wash-out period for inhaled steroids was at least 4 weeks, and for antihistamines, anti-LTs, and NSAIDs a minimum of 2 weeks. All subjects participated voluntarily and provided informed written consent under protocols approved by the local ethics committee.

Table 1 Clinical characteristics of asthmatics and healthy controls (HC)-overview

	Asthmatics	HC
Subjects, *n*	12	13
Sex (F/M)	7/5	7/6
Age (mean, [range]), years	29 (22–42)	29 (23–46)
FEV1% (mean ± SD)	100 ± 12	105 ± 9
Metacholine challenge PD20 (mean ± SD)	430 ± 558	0
Phadiatop (% positive)	100	All negative
Allergen challenge SQ PD20 (mean ± SD)	861 ± 917	ND
Allergen challenge, maximal drop in FEV 1% (mean ± SD)	31 ± 12	ND
Additional subjects, *n*	3	
Sex (F/M)	2/1	
Age (mean, [range]), years	21 (18–23)	
FEV 1% (mean ± DS)	99 ± 9	
Phadiatop (% positive)	100	

F, female; M, male; FEV1%, forced expiratory volume in one-second – percent of predicted normal; SQ, arbitrary units relating to the concentration of allergen; PD20, provocative dose causing a 20% fall in FEV1; SD, standard deviation; ND, not done.

Collection of BALF

All bronchoscopies with BAL (five aliquots of 50 ml PBS) were performed outside the pollen season as described (18). All individuals underwent a medical examination at least 2 weeks prior to BAL, including chest radiographs and blood test. BALF was stored at −80°C. A minimum of 3 weeks after the first BAL, 12 asthmatics were subjected to bronchoprovocation with individually adapted birch pollen extract doses (Aquagen SQ, ALK, Copenhagen, Denmark) as described (19) until reaching a final drop in $FEV_1 \geq 20\%$ from the postdiluent baseline according to previously described protocol values (20). BAL was performed 24 h after allergen challenge.

Analysis of BAL cells

Bronchoalveolar lavage fluid was strained through a Dacron net (Millipore, Bedford, Ireland) and centrifuged at 400 ***g*** for 10 min at 4°C. BAL cells were counted and analyzed for viability by Trypan blue exclusion (93% (84–98%) [mean (range)] for before samples, 90% (82–98%) for after samples, and 94% (86–100%) for controls). Total and differential cell counts were performed as previously described (21).

Exosome isolation

Bronchoalveolar lavage fluid exosomes from the first incoming patients ($n = 8$) and healthy individuals ($n = 9$) were purified by differential centrifugation at 100 000 ***g*** for 1 h 10 min as previously described (14). As increased centrifugation time and speed yielded more exosomes, remaining BALFs (healthy $n = 4$, asthmatics $n = 7$) were purified by centrifugation at

Table 2 Asthmatic Individuals – patient characteristics and sample collection

Patient	Symbol	Sex	Age (years)	IgE kIl/1 birch	IgE kU/l timothy	IgE kU/l others	Late allergic reaction*	BALF B/A (ml)
1	△	F	42	8	0	0	None	100/90
2	◆	F	38	47	12	Cat 11	24–49% at 3 h†	100/21
3	□	F	36	31	5	Ragweed 3	None	100/100
4	○	M	22	20	0	Dog 8	None	100/100
5	▲	F	29	51	0	Ragweed 1	21% at 6 h	100/80
6	◇	F	32	4	7	Horse 12	None	100/100
7	♦	F	34	3	0	Cat 8	None	100/100
8	✱	M	25	54	20	Cat 2	18% at 7 h	100/100
9	×	F	36	28	17	Cat 13	None	100/100
10	⊠	M	24	2	1	Mites 1	None	100/100
11	★	M	24	16	2	Ragweed 1	None	100/100
12	⚲	M	24	>100	>100	Horse > 100, cat 20	None	100/100
Additional subjects								
13	◖	F	21	>100	47	Cat 14, dog 2, fish 6, mite 1	NC	100/ND
14	➤	M	23	20	0	Cat 7, horse 1, dog 1	NC	100/ND
15	∞	F	18	5.6	4	Cat < 1, horse < 1, dog 2	NC	100/ND

*This subject received steroids (betamethasone) for her late allergic reaction.

†Drop in peak expiratory flow rate at hours postchallenge.

NC, not challenged; B/A, before/after allergen provocation; BALF, bronchoalveolar lavage fluid; F, female; M, male.

100 000 ***g*** for 2 h as described elsewhere (15). No phenotypic differences (flow cytometry markers as below) were noticed between MHC-class II-selected BALF exosomes from the two different purification protocols, and 2-h spins were performed on equal numbers of donors (four) in the comparisons between groups. Hence, data from the two protocols were pooled. Protein concentration of the resuspended pellet preparation was measured by D_C protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Flow cytometry

Bronchoalveolar lavage fluid exosomes (corresponding to 6 ml of original BALF) were coated on one μl (2.8×10^5) of anti-MHC-class II (Clone HKB1) Dynabeads (Dynal®, Oslo, Norway) and stained with FITC-labeled mAb to HLA-DR (clone L243), HLA-ABC (G46–2.6), CD63 (H5C6), CD86 (2331), MUC-1 (HMPV) (BD Bioscience, San Jose, CA), CD36 (FA6.152) (Beckman Coulter, Marseille, France), and PE-labeled mAb to CD54 (HA58), CD81 (JS-81) and compared with isotype-matched controls (BD bioscience) and phenotyped by flow cytometry using a FACS Calibur as previously described (14).

Western blot

Exosomes (15 μg) were denatured in Laemmli buffer (Bio-Rad Laboratories) and boiled at 95°C for 5 min. Samples were separated on 4–20% SDS polyacrylamide gels (BioRad Laboratories), and rabbit in-house antisera were used to immunoblot for LTA_4H, LTC_4S, FLAP, 15-LO-1, and 5-LO. Rabbit polyclonal antibodies and goat anti-rabbit antibodies were used for blotting against CD81 and β-actin (Santa Cruz Biotechnology, CA, USA), and blots were developed using ECL developing kit (Amersham Bioscience, Piscataway, NJ, USA).

Co-cultures of BEC and BALF exosomes

The human bronchial epithelial cell (BEC) line 16HB14o- (22) was kindly provided by Dr D. Greunert, University of California, San Francisco. Cells were cultured in T-25 flasks precoated with human fibronectin and bovine collagen (BD biosciences). Cells were cultured in minimal essential medium supplemented with 10% fetal calf serum (exosome depleted at 100 000 ***g*** overnight), glutamine (200 mM), and penicillin (50 mg/ml) and kept at 37°C with 5% CO_2. After reaching confluence, cells were detached using Trypsin–EDTA solution (R&D Systems, Minneapolis, MN). BALF exosomes corresponding to 1.5 ml of original BALF were added to 50 000 BEC in 250 μl medium in 24-well plates in duplicate (48 h). Supernatants were stored at −20°C before analyzed for cytokine and LT content as described below. Viability was controlled by estimating confluence, which always exceeded 90%.

ELISA for IL-6, IL-8 and HPLC analysis of LTs in cell supernatants

IL-6 and IL-8 contents in supernatants were analyzed by ELISA in duplicate according to manufacturer's instructions (Biolegend, San Diego, CA). Levels of LTs were quantified by reverse-phase HPLC (RT-HPLC) as described (23).

Activity assays for LT-producing enzymes in exosomes

Exosomes (25 μg) were incubated in PBS with 20 μM LTA_4 for 5 min at 37°C. Reactions were stopped by adding 100% methanol containing 250 pmol PGB_2 as internal standard, and chilling on ice. Samples were incubated at −20°C overnight and centrifuged (4°C, 10 000 ***g***, 10 min). Supernatants were analyzed by RP-HPLC for LTC_4 and LTB_4 as described (24).

Blocking experiments with Montelukast

Because of occasional decrease in viability during the culture of frozen cells, the culture protocol was slightly changed for blocking experiments. BEC (25 000/250 μl medium/well) were first cultured alone on fibronectin-/collagen-precoated (BD bioscience) 48-well plates at 37°C for 24 h. New medium and BALF exosomes (1 μg/well) were added in duplicate with or without the $CysLT_1$ receptor antagonist Montelukast (100 nM, Cayman Europe). Supernatants were collected after 24 h and analyzed for IL-8 as described above.

Statistical analysis

Mann–Whitney *U*-test was used for comparison between controls and asthmatics, and Wilcoxon matched-pairs signed-ranks test for before and after allergen challenge and Montelukast blocking experiments. *P*-values were considered significant at $P < 0.05$. Correlations were assessed by Spearman's correlation coefficient. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA).

Results

Increased levels of eosinophils in BALF after allergen provocation

In BALF, the number of eosinophils increased in patients after allergen provocation compared with before (Fig. 1A, also when excluding the outlier, patient 2, $P = 0.014$), with no differences in numbers of macrophages, mast cells or total cells (Fig. 1B–D). Although not significant, there was a tendency toward increased exosomal protein concentration after allergen challenge compared with before (Fig. 1E, $P = 0.092$). No differences were detected in cellular or exosomal content in BALF between healthy individuals and asthmatics, and no correlation was seen between levels of exosomal protein and either specific cell count or total cell concentration (data not shown).

Altered BALF exosome profile in asthmatics

Bronchoalveolar lavage fluid exosomes coated on anti-MHC-class II Dynabeads were analyzed by flow cytometry. BALF exosomes from asthmatics showed higher levels of the tetraspanins CD81 and CD63 (Fig. 2A,B), CD36 (Fig. 2C), and a tendency toward increased expression of HLA-DR (Fig. 2D, $P = 0.087$) compared to controls. Allergen provocation

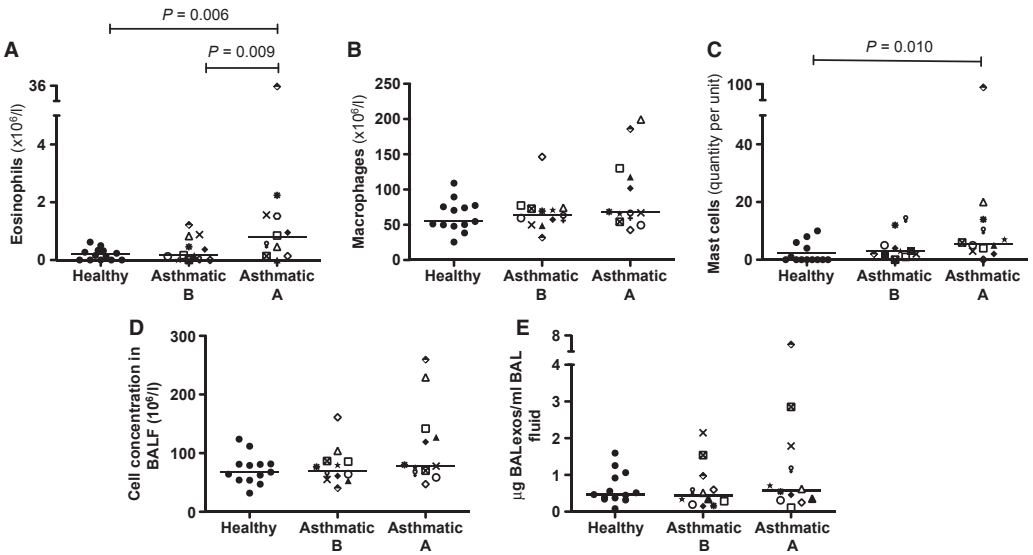

Figure 1 Analysis of bronchoalveolar lavage fluid (BALF) cells and exosomal protein. Levels of (A) eosinophils, (B) macrophages (10^6/l BALF), (C) mast cells (number of mast cells counted on at least 10 visual fields at a 160× magnification on slides), (D) total cell concentration (10^6/l BALF), and (E) exosomal protein concentration (μg/ml BALF) from BALF of healthy individuals ($n = 13$) and asthmatics (before ('B'), after ('A') allergen provocation, $n = 12$). Symbols represent different individuals (Table 2). Bars show the median values for all individuals. Significant differences between healthy and asthmatic individuals were assessed using Mann–Whitney *U*-test, and differences before and after allergen challenge using Wilcoxon matched-pairs signed-ranks test.

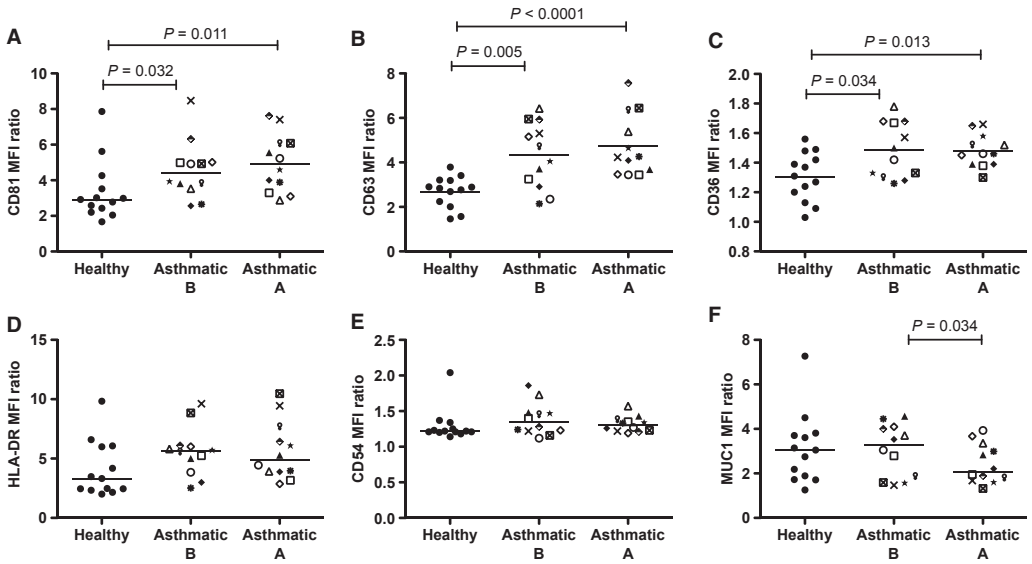

Figure 2 Increase in exosome-associated proteins on bronchoalveolar lavage fluid (BALF) exosomes from asthmatics compared to healthy individuals. BALF exosomes from healthy (n = 13) and asthmatic individuals (n = 12) were analyzed by flow cytometry. Results show MFI ratio values for (A) CD81, (B) CD63, (C) CD36, (D) HLA-DR, (E) CD54, and (F) MUC1 normalized to isotype controls. For symbol description, see Table 2. Before ('B') and 24 h after ('A') allergen provocation. Bars show the median (A, D–F) and mean values (B, C) for all individuals. Significant differences between healthy and asthmatic individuals were assessed using Mann–Whitney U-test, and differences before and after allergen challenge using Wilcoxon matched-pairs signed-ranks test.

caused no phenotypic alteration, except for MUC1, which decreased after allergen challenge (Fig. 1F). HLA-ABC and CD86 expression was consistently low, with no differences between groups (data not shown). Furthermore, a correlation was seen between HLA-DR and CD81 and CD63 on BALF exosomes (Fig. 3A,B). In conclusion, BALF exosomes from asthmatics exhibit an altered exosome profile compared to healthy subjects, even in the absence of clinical inflammation.

BALF exosomes contain functional proteins of the LT pathway

Western blot on BALF exosomes from healthy controls and asthmatics showed the presence of LTA_4H, LTC_4S, FLAP, and 15-LO-1 (Fig. 4A). FLAP, an AA-binding protein, was abundant in all BALF exosomes analyzed, whereas 5-LO was undetectable or very weak. Enrichment of CD81 was seen on BALF exosomes from patients compared to healthy individuals by analyzing the intensity of the bands normalized to loading control (β-actin) (data not shown, P = 0.023). To verify the enzyme activity, we incubated BALF exosomes (healthy, n = 2) with LTA_4 and measured LTB_4 and LTC_4. Results show that in the presence of exosomes, LTA_4 is efficiently converted to LTB_4 and LTC_4, thus proving enzyme activity (Fig. 4B).

BALF exosomes increase LT and IL-8 production in bronchial epithelial cells

To investigate the potential contribution of BALF exosomes to airway inflammation, we co-incubated BALF exosomes with BEC for 48 h and measured the production of LTs, IL-6 and IL-8. BALF exosomes from asthmatics induced significantly higher levels of LTs in BEC (mainly LTC_4, with LTB_4 below detection limit in most samples) compared to healthy controls, with no difference after allergen challenge (Fig. 5A). Furthermore, exosomes from asthmatics induced significantly higher levels of IL-8 release in BEC than those from healthy individuals, with no differences before and after allergen challenge (Fig. 5B). Similar results for IL-8, with higher activation by asthma exosomes, were obtained when adding the same amount of BALF exosomal protein (1 μg/50 000 BEC, data not shown, P = 0.013). We also detected a modest increase in IL-6 production in BEC, but with no significant differences between asthmatics and healthy individuals (data not shown). Taken together, our data suggest that BALF exosomes from asthmatics can promote LT- and cytokine release from airway epithelial cells, both during steady state and after allergen challenge.

Montelukast partially blocks exosome-induced production of IL-8 in BEC

Because BALF exosomes from most patients induced IL-8 release in BEC (Fig. 5B), we wanted to investigate whether LTs could be involved in this induction. We therefore selected BALF exosome samples previously shown to induce IL-8 release (Fig. 5B). Owing to insufficient exosome material in most patients, BALF exosomes from 3 newly recruited asthmatics were also included. BECs were co-cultured with BALF exosomes for 24 h in the presence or absence of the $CysLT_1$ receptor antagonist Montelukast. BALF exosomes

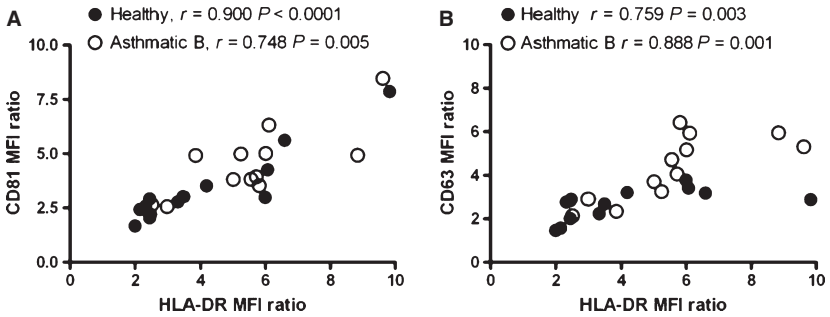

Figure 3 Correlation between HLA-DR expression and tetraspanins on bronchoalveolar lavage fluid (BALF) exosomes. (A) Correlation analysis on the expression of HLA-DR and CD81 or (B) HLA-DR and CD63 on BALF exosomes from healthy individuals (•) (n = 13) and asthmatic individuals (O) (n = 12) before allergen provocation. Exosomes were analyzed by flow cytometry, and values are presented as MFI ratio values normalized to isotype controls. Correlation analysis was assessed by Spearman's correlation coefficient (r) and associated P-values.

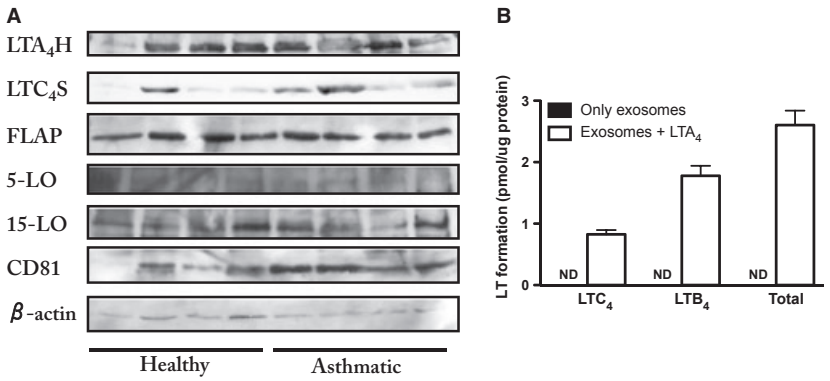

Figure 4 Bronchoalveolar lavage fluid (BALF) exosomes contain active LT-producing enzymes. (A) WB for LTA_4H, LTC_4S, FLAP, 5-LO, 15-LO-1, CD81, and β-actin (loading control), using 15 μg total exosomal protein from healthy (n = 4) and asthmatics before allergen provocation (n = 4). Representative results from three separate experiments are shown. (B) LT formation by BALF exosomes from healthy individuals (n = 2). Conversion of LTA_4 to LTB_4 and LTC_4 (pmol/μg total protein) was determined by reverse-phase HPLC (RP-HPLC). Results show mean ± SEM. ND = not detectable.

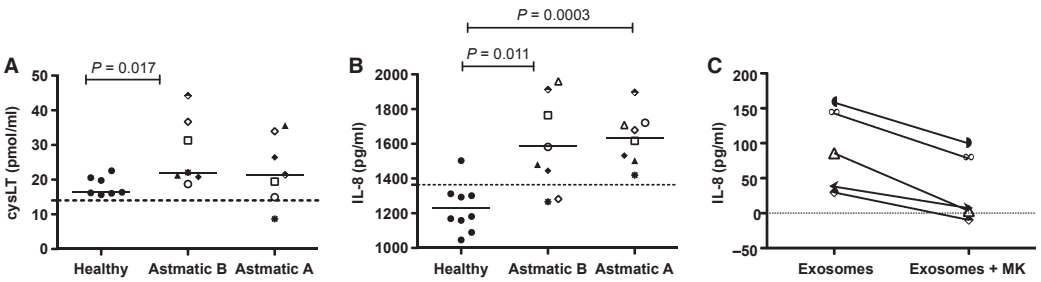

Figure 5 Bronchoalveolar lavage fluid (BALF) exosomes from asthmatics induce LTs and IL-8 release from BEC. BALF exosomes corresponding to 1.5 ml of BALF were added to 50 000 BEC in 250 μl medium for 48 h. Results show the levels of (A) LTC_4 (healthy n = 7, asthmatics Before 'B'/After 'A' n = 7) and (B) IL-8 (healthy n = 9, asthmatics B/A n = 8) in cell supernatants (pg/ml). Dotted line shows the levels of LTC_4 and IL-8 by unstimulated BEC. (C) Exosomes (1 μg) from asthmatics B (n = 5) were co-incubated with BEC for 24 h in the absence/presence of $CysLT_1$ receptor antagonist Montelukast (MK, 100 nM). Results are shown as mean of duplicates normalized to background (BEC + BALF exosomes (±MK) minus BEC alone (±MK). For symbol description, see Table 2. Mann–Whitney U-test was used to assess significant differences between healthy and asthmatic individuals, and Wilcoxon matched-pairs signed-ranks test to assess the differences between before and after allergen challenge and for Montelukast blocking experiments.

from all five patients tested induced IL-8 secretion in BEC, which could be efficiently reduced by Montelukast treatment (Fig. 5C). Our results suggest that BALF exosome–triggered IL-8 release in BEC is at least partly LT dependent.

Discussion

We have characterized and functionally compared BALF exosomes from healthy individuals with those from patients with allergic asthma. BALF exosomes from asthmatics showed an altered exosome profile and exerted pro-inflammatory functions *in vitro*, indicating potential contributions of exosomes to asthmatic inflammation.

Several surface molecules on MHC-class II-selected exosomes were up-regulated in patients compared to controls, including CD63 and CD81. This could be due to the enrichment of the detected protein on exosomes or due to an increase in MHC-class II expressing exosomes in patients. The observed tendency toward higher levels of MHC-class II on exosomes from asthmatics supports the latter and might reflect increased levels of BALF exosomes with antigen-presenting capacity. The correlation seen between tetraspanins and MHC-class II on BALF exosomes confirms previous findings (15) and is logical because tetraspanins help stabilizing the MHC complex. Interestingly, BALF exosomes express the scavenger receptor CD36, with higher expression in patients compared to controls. CD36 has been implicated in bacterial recognition (25), and it is tempting to speculate that these exosomes could play a role in asthma exacerbations in response to bacterial infections.

Characterization of BALF cells and cytokines from the individuals in this study has previously been published (19). This study showed the increased levels of $Foxp3^+$ T cells and pro-inflammatory (IL-5, IL-6, IL-9) and anti-inflammatory cytokines (IL-10) in BALF after allergen provocation. We found no correlation between levels of cytokines and exosomal markers (data not shown), possibly due to limited patient material and/or differences in kinetics. It would be interesting to study earlier time-points for exosome content. Noteworthily, allergen challenge only caused a mild inflammation in this study, and it would be interesting (but ethically challenging) to study more severe cases of allergic asthma as well as to follow patients during pollen season. Of note, patient no 2 (Table 2) experienced a late-phase reaction and showed the highest levels of inflammatory cells and exosomal protein (Fig. 1A–E), displayed among the highest levels of surface molecules (Fig. 2A–C), and induced among the highest levels of IL-8 in BEC (Fig. 5B).

Leukotrienes are key players in asthma pathogenesis, and antagonists are clinically beneficial. We found enzymes for LT biosynthesis in BALF exosomes from healthy individuals and asthmatics. To verify the enzyme activity, we tested the ability of BALF exosomes from healthy individuals to convert LTA_4 into LTB_4/LTC_4 (Fig. 4B). Unlike APC-derived exosomes (17), BALF exosomes synthesized higher levels of LTB_4 compared to LTC_4, suggesting a specific eicosanoid profile. Owing to material limitations, we were not able to perform comparative studies on enzyme activity between BALF exosomes from controls and asthmatics. Nevertheless, activity of LTA_4H and LTC_4S in BALF exosomes suggests that exosome-assisted production of LTs could be of importance for lung immunity and inflammation. In our parallel experiments published elsewhere, BALF exosomes could generate 50 times more LTs from LTA_4 compared to plasma exosomes (17), suggesting that BALF exosomal LTs might be relevant in airway inflammation and host defense also in healthy individuals.

Earlier studies have reported the presence of soluble LTA_4H in human BALF with LTB_4-producing capacities (26). It appears possible that this could partly be exosome mediated. We further speculate that exosomal LTA_4H and LTC_4S could potentially amplify the generation of inflammatory LTs, particularly in an inflammatory context with infiltration of activated, LTA_4-secreting neutrophils (27). Presence of cPLA2 was not determined in BALF exosomes, but has previously been reported for mast cell exosomes (28). It is likely that BALF exosomes also contain cPLA2 that can generate AA. However, LT biosynthesis might also occur through transcellular mechanisms.

Both primary BEC and the cell line 16HB14o- have been shown to produce cytokines and LTs (29). We show that BALF exosomes from asthmatics can increase the production of LTs and IL-8 in BEC (Fig. 5A–B), whereas control exosomes did not induce (or may even suppress) a response. We also found that BALF exosomes generate more LTB_4 compared to LTC_4 (Fig. 4B), while BEC and BALF exosome co-cultures mainly generated LTC_4 production (Fig. 5A). This suggests that LT production is largely generated by BEC. Furthermore, we did not observe LTA_4 release by BEC, and thus, we hypothesize that BALF exosomes might contribute to intracellular LT biosynthesis in phagosome-like structures (30) or stimulate LTC_4 production, for example, via cytokines carried within exosomes or through surface molecules, such as CD36 (31).

Clinical studies have shown LT modifiers to be beneficial in asthma treatment. Montelukast is a potent $CysLT_1$ receptor antagonist, which ameliorates symptoms of asthma (32). By treating BEC with Montelukast, we show that the induction of IL-8 in response to BALF exosomes might be partly LT dependent. Interestingly, inhibition of IL-8 release by Montelukast was recently demonstrated for monocytes (33) and epithelial cells (34). However, because IL-8 production was only partially blocked by Montelukast, we argue that also other than LT effects are likely to operate in asthma.

We speculate that the BALF exosomes analyzed partly originate from alveolar epithelial cells because of their expression of the epithelial marker MUC1 in combination with HLA-DR (35). On the other hand, activated DC have also been reported to express MUC1 (36) and could thus be a potential cellular source for exosome production in BALF. A decrease in MUC1 expression after allergen provocation (Fig. 2F) could be reflecting a change in the production of

epithelial cell–derived exosomes and/or a change in DC activation. Accordingly, our inability to detect a correlation between specific cell count and levels of exosomes in BALF suggests that other cells apart from those analyzed in BALF, such as lung epithelial cells or DC, might contribute to exosome production. Alveolar macrophages, which are particularly potent LT producers (37), are also likely to contribute to BALF exosome production. Dissecting the specific eicosanoid fingerprint of BALF exosomes might in the future shed light into the cellular origin of BALF exosomes and could provide clues to changes in cellular compositions in, for example, patients with lymphoma or lung cancer. Thus, the eicosanoid profile of exosomes might be a promising novel biomarker of inflammation.

Interestingly, we found similar features of BALF exosomes from asthmatics as those previously seen from patients with sarcoidosis (15), such as increased surface molecules and the capacity to increase IL-8 production in BEC. This suggests a general alteration in BALF exosomes during inflammation. However, our data also suggest an intrinsic alteration in BALF exosomes during nonclinical inflammatory conditions. The inability to detect changes after allergen challenge could partly be due to limited patient material, the mild asthmatic response, and/or the specific time-point chosen for the collection of BALF (24 h).

Even though our data show that exosomes from BALF can induce cytokine and LT responses *in vitro*, it is not possible from this study to conclude that exosomes contribute to asthma pathogenesis. However, we believe that the possibility for exosomes to concentrate at specific sites or on certain cells might lead to a high concentration locally. This would lead to potential cytokine and leukotriene release in response to exosomes during disease. Whether exosomes are most relevant in acute or chronic asthma needs further investigation. In conclusion, our data suggest that BALF exosomes might contribute to asthma pathogenesis by inducing pro-inflammatory cytokines and LT generation in the lung. This could have implications in the development of future therapies targeting exosome production.

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License for publication

This work has not been published before and has been read and approved by all authors.

Ethical approval

The local ethics committee has approved this study, and informed written consent was obtained from all subjects.

Authors' Contributions

P.T.P and J.E. designed the experiments and were involved in acquiring and analyzing data. P.T.P., J.E., C.A., A.L., and Q.K.R. performed the experiments. R.G. was involved in patient selection. M.N., O.R., R.G., J.G., A.E., A.S., and S. G. were involved in the study design. A.E. performed BAL. P.T.P., J.E., A.S., and S.G. prepared the manuscript. C.A., M.N., O.R., J.G., and A.E. edited the manuscript.

Conflict of interest statement

None declared.

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