

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Eosinophil-derived exosomes contribute to asthma remodelling by activating structural lung cells

J. A. Cañas^{1,2} | B. Sastre^{1,2} | J. M. Rodrigo-Muñoz¹ | M. Fernández-Nieto^{2,3} |
P. Barranco^{2,4} | S. Quirce^{2,4} | J. Sastre^{2,3} | V. del Pozo^{1,2} 

¹Department of Immunology, IIS-Fundación Jiménez Díaz, Madrid, Spain

²CIBER de Enfermedades Respiratorias (CIBERES), Madrid, Spain

³Department of Allergy, IIS-Fundación Jiménez Díaz, Madrid, Spain

⁴Department of Allergy, Hospital La Paz-Institute for Health Research (IdiPAZ), Madrid, Spain

Correspondence

Victoria del Pozo, Immunology Department, IIS-Fundación Jiménez Díaz, Madrid, Spain.
Email: vpozo@fjd.es

Funding information

Fondo de Investigación Sanitaria—FIS, Grant/Award Number: PI15/00803; CIBER de Enfermedades Respiratorias (CIBERES); a Carlos III Institute of Health Initiative; Conchita Rábago Foundation (FCR); FEDER funds

Summary

Background: Eosinophils, a central factor in asthma pathogenesis, have the ability to secrete exosomes. However, the precise role played by exosomes in the biological processes leading up to asthma has not been fully defined.

Objective: We hypothesized that exosomes released by eosinophils contribute to asthma pathogenesis by activating structural lung cells.

Methods: Eosinophils from asthmatic patients and healthy volunteers were purified from peripheral blood, and exosomes were isolated from eosinophils of asthmatic and healthy individuals. All experiments were performed with eosinophil-derived exosomes from healthy and asthmatic subjects. Epithelial damage was evaluated using primary small airway epithelial cell lines through 2 types of apoptosis assays, that is, flow cytometry and TUNEL assay with confocal microscopy. Additionally, the epithelial repair was analysed by performing wound healing assays with epithelial cells. Functional studies such as proliferation and inhibition-proliferation assays were carried out in primary bronchial smooth muscle cell lines. Also, gene expression analysis of pro-inflammatory molecules was evaluated by real-time PCR on epithelial and muscle cells. Lastly, protein expression of epithelial and muscle cell signalling factors was estimated by Western blot.

Results: Asthmatic eosinophil-derived exosomes induced an increase in epithelial cell apoptosis at 24 hour and 48 hour, impeding wound closure. In addition, muscle cell proliferation was increased at 72 hours after exosome addition and was linked with higher phosphorylation of ERK1/2. We also found higher expression of several genes when both cell types were cultured in the presence of exosomes from asthmatics: *CCR3* and *VEGFA* in muscle cells, and *CCL26*, *TNF* and *POSTN* in epithelial cells. Healthy eosinophil-derived exosomes did not exert any effect over these cell types.

Conclusions and Clinical Relevance: Eosinophil-derived exosomes from asthmatic patients participate actively in the development of the pathological features of asthma via structural lung cells.

KEYWORDS

asthma, bronchial smooth muscle cells, eosinophils, epithelium, exosomes, lung

1 | INTRODUCTION

Asthma is a heterogeneous, chronic inflammatory disease of the airways characterized by wheezing, breathlessness and chest tightness, and it is associated with variable airflow obstruction, airway hyperresponsiveness, persistent Th2-driven airway inflammation and remodelling.¹ Chronic airway remodelling is one of the most important characteristic processes of asthma pathology and involves structural lung cells (fibroblasts, bronchial smooth muscle cells [BSMC] and airway epithelial cells) and effector cells such as Th2 lymphocytes, mast cells and eosinophils. Effector cells can release pro-contractile stimuli and pro-inflammatory mediators, such as interleukin [IL]-4, IL-9 and IL-13, inducing mucus hypersecretion and goblet cell metaplasia and increasing airway hyperresponsiveness.²

Airway remodelling includes alterations of airway epithelium, subepithelial collagen and extracellular matrix deposition, and an increase in bronchial smooth muscle mass.³ This process results in airway wall thickening, which contributes to inflammation of the airways, longer duration of damage and inflammation and, consequently, greater asthma severity.

Airway smooth muscle (ASM) cells make up the smooth muscles of the airway wall. Asthma development implies contraction, proliferation, hypertrophy, hyperplasia and migration of these cells, which cause airway obstruction as well as tissue remodelling.⁴ In these cells, hyperplasia may be a consequence of increased proliferation and survival and may also be associated with intrinsic abnormalities and interactions with inflammatory molecules.⁵ However, these cells not only act as contractile elements; different studies have shown that muscle cells of the airways can participate in the vascular remodelling of the airways during the asthmatic process, secreting cytokines, growth factors, matrix proteins and other pro-inflammatory mediators.⁶

Asthmatic processes do not only involve muscle cells; rather, epithelial cells also participate, and these are capable of releasing different factors that could play an important role in the onset of damage.

The bronchial epithelium is the most significant barrier against external damage such as that caused by allergens, making it an essential defence mechanism. Epithelial desquamation and bronchial muscle hyperplasia combined with angiogenesis give rise to bronchial remodelling processes that lead to chronic damage with persistent airflow limitation.⁷ This occurs in patients with long-lasting disease, and treatments such as inhaled corticosteroids seem to be ineffective in reversing or preventing this feature of asthma.⁸ Also, airway epithelium and smooth muscle cells are implicated in other processes related to airway remodelling, such as mucus production and induction of the innate immune response by the activation of dendritic cells and Th2 cells to release mediators linked to remodelling.⁹ Damaged cells have increased expression and secretion of several cytokines and chemokines, such as thymic stromal lymphopoietin (TSLP), IL-25, IL-33, chemokine (C-C motif) ligand (CCL) 1 and CCL24, which amplify Th2 response and promote the

recruitment of several cell types towards the airways, including eosinophils.

Eosinophils are important effector cells in asthma that act by releasing granules containing multiple inflammatory factors. Some of these granules are exosomes, which are extracellular nanovesicles that are small in size (30–120 nm) and contain multiple molecules, such as nucleic acids (DNA, microRNAs [miRNA], RNA), proteins and lipid mediators.¹⁰ Exosomes, which act as independent extracellular functional units, have been confirmed to be an important and active intercellular communication tool, playing key roles in physiological processes as well as the pathogenesis of several inflammatory diseases,¹¹ including asthma.¹²

In previous studies, we demonstrated that asthmatic eosinophils may release a greater quantity of exosomes than that seen in healthy volunteers.^{13,14} Moreover, we have recently shown that these asthmatic eosinophil-derived exosomes bring about functional changes in eosinophils¹⁴ in the context of asthma pathology. As in eosinophils, these structures may also exert a variety of effects on lung structural cells, therefore influencing the development and course of the disease.

The aim of this study was to determine, in the context of asthma development, the effect of eosinophil-derived exosomes on small airway epithelial cells (SAEC) and BSMC, both of which are implicated in airway remodelling.

2 | MATERIALS AND METHODS

2.1 | Subjects

Asthmatics ($n = 19$) and healthy subjects ($n = 9$) were recruited from the Allergy Department of IIS-Fundación Jiménez Díaz and Hospital La Paz-Institute for Health Research (IdiPAZ) (Madrid) as well as the staff of both hospitals. All subjects were properly informed and signed informed consent. The study was conducted in accordance with the principles set forth in the Declaration of Helsinki and was approved by the Fundación Jiménez Díaz Ethics Committee. The clinical and epidemiologic characteristics of patients are shown in Table 1.

Asthma patients had a consistent history of asthma.¹⁵ None had a history of respiratory tract infections for at least the 6-week period preceding the study. We included both atopic and non-atopic asthmatic subjects because there were no differences in the parameters assessed between the 2 sets of patients. For patients who were receiving inhaled corticosteroids, the drugs were withdrawn for at least 2 weeks before the samples were obtained. No patient was receiving oral corticosteroids (for at least 6 months before the study), leukotriene receptor antagonists, aspirin or any other cyclooxygenase inhibitor.

2.2 | Recombinant proteins and reagents

Recombinant human IL-5 and GM-CSF were acquired from R&D Systems (Minneapolis, MN, USA). FcR Blocking Reagent and anti-

TABLE 1 Clinical and epidemiological characteristics of asthmatic patients and healthy subjects

	Asthma (n = 19)	Healthy (n = 9)
Age (y) ^a	38.84 (24-47)	33.44 (27-51)
Male (%)	3 (15.79)	2 (22.22)
Atopy (%)	16 (84.21)	3 (33.33)*
Smoking history		
Current (%)	5 (26.32)	1 (11.11)
Ex-smoker (%)	4 (21.05)	1 (11.11)
Non-smoker (%)	10 (52.63)	7 (77.78)
FEV1/FVC (%) ^b	75.72 (58.32-84.55)	ND
FENO (ppb) ^a	38.93 (5.80-110.00)	ND
Total serum IgE (kU/L) ^b	135.00 (18.60-1339.00)	ND
Eosinophils (%) ^a	5.70 (3-10.30)	<3.00

NA, not available; FEV1, forced expiratory volume in first second; FVC, forced vital capacity.

^aMean (range).

^bMedian (range).

**P* < .05.

human SIGLEC8-FITC were purchased from MACS Miltenyi Biotec (Bergisch Gladbach, Germany), and CD16-PE flow cytometry antibodies (Ab) were supplied from Becton Dickinson (New York, NY, USA). Ab against ERK 1/2, phosphorylated-ERK 1/2 (pERK1/2), phosphorylated-AKT (pAKT), phosphorylated-STAT3 (pSTAT3) and β -actin were obtained from Cell Signalling Technology (Beverly, MA, USA), and α -tubulin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PD98059, an ERK inhibitor, was acquired from Sigma-Aldrich (Saint Louis, MO, USA). Fluorescent lipophilic linker PKH67 was also obtained from Sigma-Aldrich.

2.3 | Eosinophil isolation

Eosinophils from healthy and asthmatic subjects were purified as previously described.¹⁶ For eosinophil isolation, we employed a magnetic bead separation technique, as described in the manufacturer's protocol (EasySep[®] StemCell Technologies, Vancouver, Canada). The eosinophil (SIGLEC8⁺CD16⁺ cells) purity obtained was consistently > 96%.

2.4 | Cell-culture conditions

Purified eosinophils (1×10^6 cells/mL) were cultured in complete RPMI-1640 medium Gibco[®] (Thermo Fisher Scientific, Wilmington, MA, USA) supplemented with a cocktail of IL-5 (5 ng/mL) and GM-CSF (10 ng/mL) for 24 hours. SAEC and BSMC were suspended and maintained in SABM and SmGM growth medium (Lonza, Basel, Switzerland), respectively. Cells were cultured with or without exosomes from eosinophils of asthmatic patients or healthy individuals for different times (3 hours, 6 hours, 24 hours, 48 hours and 72 hours). All cultures were maintained at 37°C in an atmosphere containing 5% CO₂.

2.5 | Exosome purification and quantification

Exosomes produced by 4×10^6 eosinophils obtained from asthmatic or healthy individuals were isolated and quantified from the cell-culture supernatants as previously described.¹³ The optimal dose of exosomes was based on previous published data,¹⁴ establishing 6×10^6 exosomes/mL as the most appropriate concentration. Several dose-response experiments in both kind of cells (SAEC and BSMC) proved that this concentration is the optimal exosome dose to have an effect on these cell types (data not shown).

2.6 | RNA isolation, RT-PCR and TaqMan gene expression assays

Total RNA was purified from BSMC and SAEC using the Chomczynski method.¹⁷ RNA was quantified, and 1 μ g of RNA was reverse-transcribed into cDNA.

Semi-quantitative real-time PCRs (qRT-PCR) were performed on a 7500 Real-Time PCR system (Applied Biosystems) using TaqMan gene expression assay probes for *GAPDH*, *TSLP*, *POSTN*, *CCL26*, *MMP9*, *VEGFA*, *TGFB1*, *TNF*, *TLR3*, *NFKB2*, *CCR3*, *EGF*, *FLG*, *IL33* and *FGF2*. All genes analysed were examined for their relative expression, which was calculated using the $2^{-\Delta\Delta Ct}$ method,¹⁸ where:

$$\Delta\Delta Ct = \Delta Ct_{\text{Exosomes}} - \Delta Ct_{\text{Control}} \text{ and } \Delta Ct = \bar{X}Ct_{\text{Gene}} - \bar{X}Ct_{\text{GAPDH}}$$

2.7 | Apoptosis assays

Apoptotic SAEC percentage was evaluated by 2 different methods: annexin V by flow cytometry in a FACSCanto II flow cytometer (Becton Dickinson) and TUNEL assay using a Leica TCS microscope (Leica Microsystems, Wetzlar, Germany) with a 20X objective to visualize them.

The percentage of apoptotic SAEC was assessed by flow cytometry using the Annexin V Apoptosis Detection Kit (eBioscience, San Diego, CA, USA). SAEC were cultured in a 24-well culture plate until 80% confluence was reached. After this, exosomes from healthy individuals and asthmatic patients were added and cells were recovered with trypsin/EDTA (Sigma-Aldrich) at different times (6 hours, 24 hours and 48 hours). Annexin V-FITC⁺/PI-PerCP-Cy5⁺ and annexin V-FITC⁺/PI-PerCP-Cy5⁺ cells were selected, defining total apoptosis percentage.

Using TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP Nick end labelling), we measured the characteristic changes in DNA fragmentation or apoptosis. SAEC seeded in 2-well chamber slides (Eppendorf, Hamburg, Germany) were incubated with 6×10^6 exosomes/mL from healthy or asthmatic individuals or ultracentrifuged medium (control) at 37°C and 5% CO₂ for 24 hours. After this, the TUNEL assay was performed using the ApopTag Fluorescence In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. As a positive control, we performed DNase I treatment on the cells. Results are

expressed as mean percentages of apoptotic cells with respect to the total DAPI⁺ cells counted in 11-25 random fields.

2.8 | Time-lapse assays (Wound healing)

A wound was made in the SAEC monolayer, and the optimal dose of exosomes from healthy or asthmatic subjects or ultracentrifuged medium (control) was added. Images were taken with a Nikon Eclipse TiE microscope (Nikon Instruments, Melville, NY, USA). Time-lapse analysis was performed with NIS-AR (Nikon Instrument) and ImageJ software. Results are expressed in terms of percentage of covered area as mean $\pm$ SD. Twenty-four fields (8 fields per assay from 3 independent experiments) for experiments with exosomes from healthy subjects and exosomes from asthmatic patients were monitored at each time-point.

2.9 | Bronchial smooth muscle cell proliferation assays

Bronchial smooth muscle cells proliferation, with and without exosomes, obtained from asthmatic and healthy subjects was evaluated with Amersham Cell Proliferation Biotrak ELISA System (GE Healthcare, Buckinghamshire, United Kingdom) following the manufacturer's instructions. Briefly, BSMC were cultured in a 96-well plate. Exosomes of eosinophils from healthy and asthmatic subjects were added, and the BSMC were incubated for 72 hours. After this period, BrdU were added and cells were incubated for 24 hours at 37°C in an atmosphere containing 5% CO₂. Later, the plate was read at 450 nm in an Infinite M200 Tecan luminometer (Tecan, Männedorf, Switzerland). The results were analysed in terms of fold induction in proliferation compared to negative controls (BSMC cultured with ultracentrifuged medium).

2.10 | pERK inhibition assay

Bronchial smooth muscle cells were cultured with PD98059, a selective ERK inhibitor, for a period of 2 hours, and after this, exosomes from asthmatic patients were added and proliferation and pERK 1/2 expression levels were evaluated at 72 hours. Results are expressed as fold induction.

2.11 | Western blot analysis

Using Western blot, we assessed the phospho-ERK1/2 pathway in BSMC and the pSTAT3 and pAKT pathways in SAEC in the presence or absence of exosomes from healthy or asthmatic individuals. Phospho-ERK1/2 data were quantified and normalized against ERK1/2 level expression. Phospho-STAT3 and pAKT were normalized against β -actin.

2.12 | Immunofluorescence analysis

Purified exosomes from healthy individuals and asthmatic patients were labelled with the fluorescent lipophilic linker PKH67 (Sigma-Aldrich) following the manufacturer's instructions.

Bronchial smooth muscle cells were seeded in an Imaging Disc G1.0 from MACS (Miltenyi Biotec) for 24 hours; SAEC were seeded in 2-well chamber slides (Eppendorf) and were incubated for 72 hours. Nuclei were stained with DAPI 300 nM (Thermo Fisher Scientific). The optimal dose of labelled exosomes (6×10^6 /mL) from the eosinophils of asthmatic subjects and healthy donors was added to the BSMC or SAEC, and confocal microscopy was performed with a Leica TCS microscope (Leica Microsystems) with a 20X objective. Images of the cells were acquired at different times. Fluorescence intensity quantification was performed with the ImageJ program.

2.13 | Statistical analysis

Results are expressed as mean $\pm$ SD. Normality was analysed using the Shapiro-Wilk test. Parametric data comparison between groups was performed using unpaired *t* test (SAEC and BSMC gene expression, SAEC and BSMC pathway analysis, and BSMC proliferation when a comparison was performed between asthmatic and healthy groups), *t* test with Welch's correction (exosome uptake by SAEC, and BSMC proliferation), or 1 sample *t* test (signalling factor analysis in SAEC and BSMC, and BSMC proliferation assay when the comparison involved negative controls, which take a value = 1). The Mann-Whitney U test was used in nonparametric data comparison between 2 groups (BSMC and SAEC gene expression assays, and exosome uptake by BSMC). Comparisons between more than 2 groups were performed using ANOVA with Bonferroni *post hoc* test for parametric data (annexin V apoptosis assay), and the Kruskal-Wallis procedure with the Dunn *post hoc* test was applied for samples with nonparametric data (wound healing and TUNEL assays). The significance level was set at an α -error level of < 5%. Differences were considered significant when $P < .05$.

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA).

3 | RESULTS

3.1 | Eosinophil-derived exosomes from asthmatics delay wound repair of SAEC

We measured the capacity of epithelial cell monolayers to repair wounds when exosomes are present in the microenvironment. Wound healing was evaluated by time-lapse assay, showing complete closure at 24 hours in controls and exosomes from healthy subjects, while in assays using exosomes from asthmatics, the wound was still not completely closed at this time-point (Figure 1A and Video S1).

At 12 hours, the control condition and cells with exosomes of eosinophils from healthy volunteers had closed over half of the wound ($55.15 \pm 25.21\%$ and $53.77 \pm 18.80\%$, respectively; Figure 1B), while cells with asthmatic eosinophil-derived exosomes had not yet reached 50 percent closure ($38.61 \pm 22.36\%$; $P < .05$). At 24 hours, the controls and the cells cultured with exosomes from

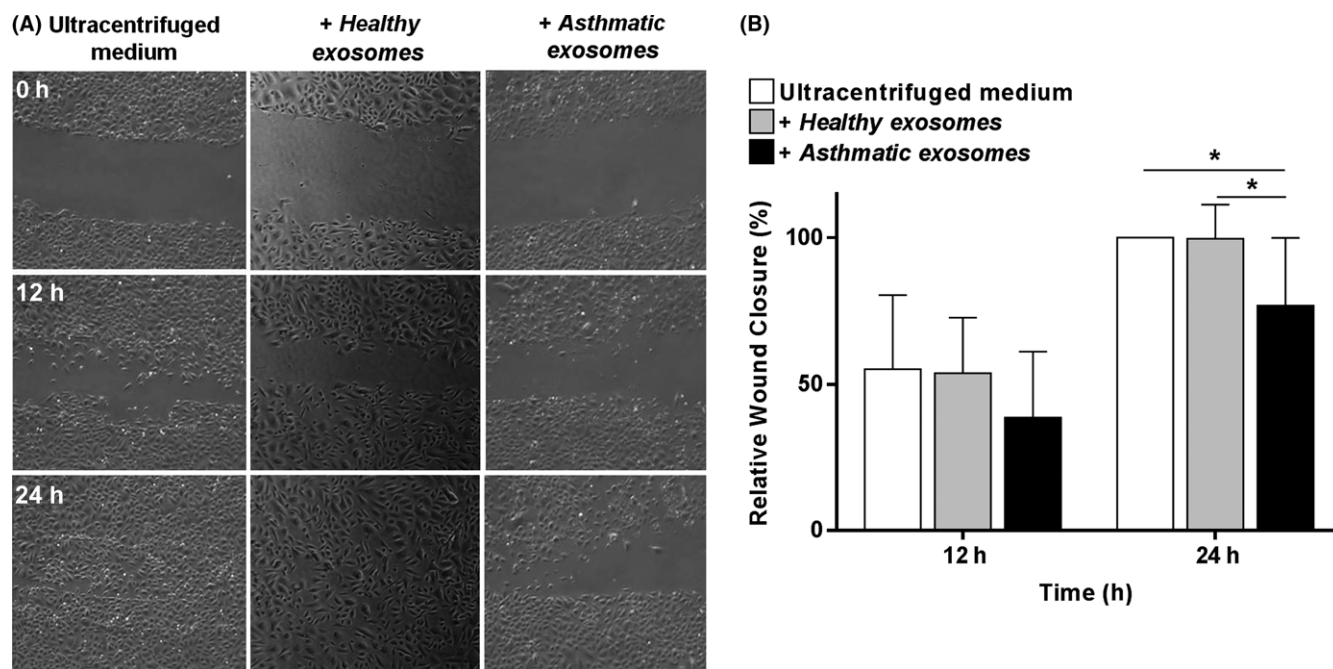


FIGURE 1 Asthmatic eosinophil-derived exosomes delay wound healing in SAEC. A, Time course of wound closure in SAEC (untreated or treated with exosomes of eosinophils from asthmatic and healthy subjects). Images were taken at different times (0, 12 and 24 h) after exosome addition. B, Relative wound closure was quantified as the relative percentage of wound area covered by cells in the presence of healthy or asthmatic eosinophil-derived exosomes compared to complete wound repair in control condition (ultracentrifuged medium) at 24 h. Data represent mean $\pm$ SD, $n = 24$; $*P < .05$

healthy subjects had completely or almost completely closed the wound (100% and $99.60 \pm 11.67\%$, respectively; $P > .05$); in the presence of exosomes from asthmatic patients, the area closed for the cells was $76.67 \pm 23.25\%$ ($P < .05$ compared with both conditions).

3.2 | Addition of exosomes enhances apoptosis rate in SAEC

A significant increase in apoptosis was observed at 24 hours when we evaluated the annexin V-positive cells after SAEC culture with exosomes from asthmatic patients. In Figure 2A, we can observe the apoptosis percentage generated by exosomes from asthmatic subjects compared to controls (SAEC cultured without exosomes) and SAEC cultured with exosomes from healthy individuals. At 6 hours and 48 hours, no significant changes in apoptosis percentage were observed; however, a great increase in apoptosis percentage was observed at 24 hours with exosomes from asthmatic patients ($21.79 \pm 9.07\%$), reaching statistical significance with ultracentrifuged medium ($8.60 \pm 3.02\%$; $P < .01$), and with cells incubated with exosomes from healthy individuals ($6.52 \pm 1.90\%$; $P < .001$). The results obtained using exosomes from healthy subjects did not show differences with regard to negative controls at any time-point ($P > .05$; Figure 2A). Representative flow cytometry dot plots for each condition are shown in Figure S1.

To confirm these results, apoptosis rate was evaluated at 24 hours by TUNEL assay, revealing similar results. Figure 2B shows

confocal microscopic images of the TUNEL assay. A higher percentage of apoptotic cells with respect to the total positive DAPI cells was observed when SAEC were incubated with exosomes from asthmatic patients ($8.65 \pm 6.14\%$) compared with cells cultured with ultracentrifuged medium ($1.33 \pm 0.55\%$) and exosomes from healthy subjects ($2.47 \pm 1.12\%$; $P < .001$). No differences were observed between ultracentrifuged medium and SAEC incubated with exosomes from healthy subjects ($P > .05$).

Both techniques allow us confirm that exosomes from eosinophils of asthmatic patients increase epithelial apoptosis and play an important role in the characteristic epithelial damage seen in asthma.

3.3 | Eosinophil exosomes increase cytokine production in SAEC

Analysis of relative gene expression in SAEC with exosomes and without wounds (simulated initial stages of asthma) showed 3 genes with a significant increase in mRNA levels compared to cells cultured with ultracentrifuged medium (1.00 ± 0.00 -fold) and with exosomes from healthy subjects: *TNF* at 24 hours (2.07 ± 0.84 -fold vs 0.78 ± 0.53 -fold, $P < .05$), *CCL26* at 6 hours (2.05 ± 0.76 -fold vs 1.28 ± 0.75 -fold, $P < .05$) and *POSTN* at 6 hours (2.00 ± 0.68 -fold vs 1.06 ± 0.74 -fold, $P < .05$; Figure 3A). No significant changes were found in the levels of *TSLP*, *NFKB2*, *TLR3*, *IL33*, *EGF* and *FLG* relative to controls (Figure S2).

When we analysed relative gene expression profile in SAEC with exosomes and wounds (simulated increased severity of asthma with

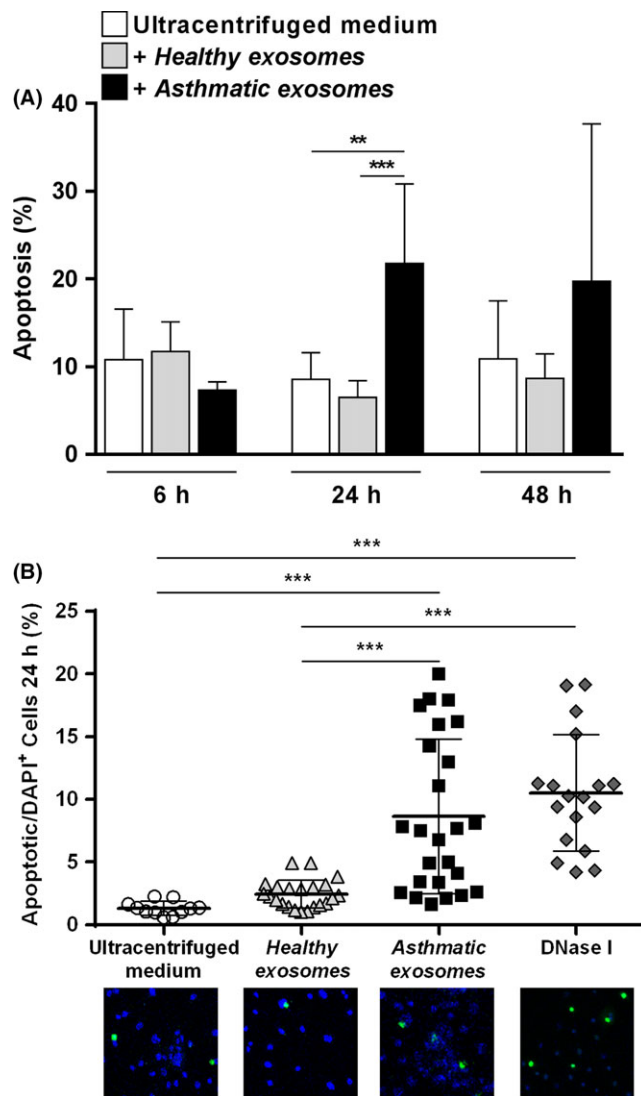


FIGURE 2 Exosomes from asthmatic eosinophils produce apoptotic effects in SAEC. A, Small airway epithelial cells were treated with an optimal dose of exosomes from both asthmatic ($n = 8$) and non-asthmatic ($n = 6$) eosinophils during different times (6, 24 and 48 h). Apoptosis levels were measured with annexin V analysis by flow cytometry. B, Apoptosis at 24 h was confirmed in SAEC cultures using TUNEL assays. Apoptosis was determined by counting apoptotic cells in 11-25 random fields. Cells were treated with DNase I solution (positive control), ultracentrifuged medium (control without exosomes) and optimal dose of exosomes from asthmatic and healthy donors. Immunofluorescence green staining represents apoptotic cells. Nuclei were stained with DAPI (blue). ** $P < .01$, *** $P < .001$

established damage), we observed a significant increase in the mRNA levels of the same genes described above, although at different times: *TNF* at 3 hours (1.87 ± 0.66 -fold vs 1.19 ± 0.16 -fold, $P < .05$), *CCL26* at 24 hours (1.91 ± 0.47 -fold vs 0.74 ± 0.10 -fold, $P < .05$) and *POSTN* at 24 hours (1.83 ± 0.51 -fold vs 0.80 ± 0.16 -fold, $P < .05$) (Figure 3B). No significant changes were found in the other genes evaluated.

No gene expression changes were observed with respect to negative controls when SAEC were cultured with exosomes from healthy subjects in both situations, with or without wounds (Figure 3).

3.4 | PI3K/AKT and JAK-STAT modulate exosome-induced changes in SAEC

The presence of exosomes from asthmatic patients produces a significant reduction in pAKT and pSTAT3 at 24 hours (0.56 ± 0.15 -fold and 0.81 ± 0.20 -fold, respectively), compared to negative controls ($P < .001$ and $P < .01$; Figure 4). However, this situation was reversed at 48 hours, and a significant increase in both phosphorylated factors was observed (1.25 ± 0.05 -fold and 1.22 ± 0.20 -fold, $P < .01$ and $P < .05$, Figure 4). When SAEC were cultured with exosomes from healthy subjects, the expression of pAKT and pSTAT3 was similar to the one obtained with negative controls at both time-points ($P > .05$; Figure 4), showing significant differences in both factors in relation to the changes obtained with exosomes from asthmatic patients at 24 hours (pAKT: 1.04 ± 0.33 , $P < .01$; pSTAT3: 1.09 ± 0.08 , $P < .01$; Figure 4). These data suggest that exosomes could modulate SAEC survival and function by regulating the PI3K/AKT and JAK-STAT signalling pathways.

3.5 | Bronchial smooth muscle cell response to exosomes

As hyperplasia and hypertrophy of muscle cells are characteristic processes of asthma, we analysed BMSC proliferation.

We observed a significant increase in proliferation 72 hours after addition of exosomes from eosinophils of asthmatic patients (1.24 ± 0.10 -fold, $P < .001$; Figure 5A); however, this effect was not observed when we cultured cells with exosomes from healthy subjects (1.02 ± 0.03 -fold, $P > .05$, Figure 5), showing similar proliferation levels to the negative controls and reaching statistical significance when we compared this with proliferation obtained with exosomes from asthmatic patients ($P < .01$).

Several studies have demonstrated the implication of the MAPK pathway in BSMC proliferation. Using Western blot, we evaluated the phosphorylation levels of ERK1/2 (MAPK/ERK elements) at 72 hours. As depicted in Figure 5B, a significant increase in pERK protein expression was detected after treatment with exosomes from asthmatic subjects at 72 hours compared to the negative controls (1.19 ± 0.14 -fold, $P < .001$) and with eosinophil-derived exosomes from healthy subjects (0.87 ± 0.32 -fold, $P < .05$, Figure 5B).

To prove that augmented levels of pERK have a direct effect on the observed changes in BSMC proliferation, we used a specific ERK inhibitor, PD98059. When we incubated BSMC with $50 \mu\text{M}$ PD98059 prior to adding exosomes from asthmatics, we observed a significant reduction in proliferation compared to BSMC incubated with exosomes from asthmatic subjects

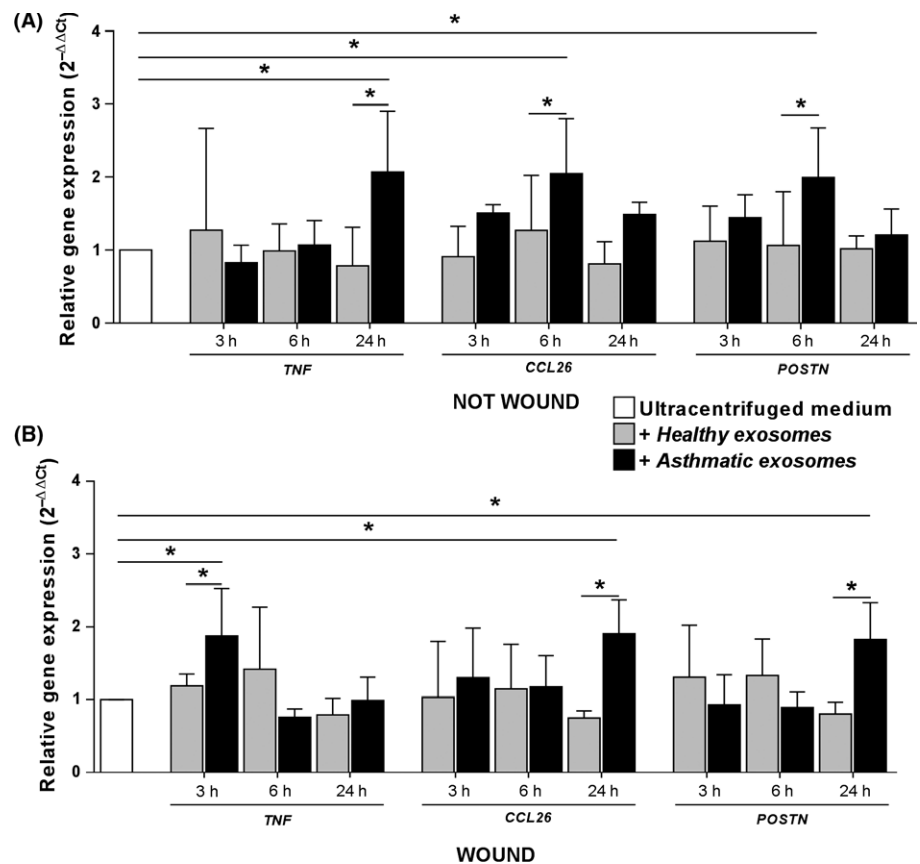


FIGURE 3 Incubation of SAEC with eosinophil-derived exosomes from asthmatics induces changes in gene expression profile. Relative gene expression from different molecules was quantified in SAEC in 2 different stages of asthma pathogenesis, without wound (early stages) and with wound in a cell monolayer (chronic stages). A, Gene expression profile of SAEC cultured without wound. B, Gene expression profile of SAEC cultured with wound in a cell monolayer. Changes were observed in the same genes as in SAEC culture without wound. All experiments were performed in triplicate. * $P < .05$, $n = 6$

(1.03 ± 0.05 -fold vs 1.24 ± 0.10 -fold, $P < .0001$; Figure 5A). A trend towards decreased expression of pERK was observed by Western blotting when we added the inhibitor prior to asthmatic exosome addition (Figure 5B).

According to these results, the MAPK pathway is implicated in the increased BSMC growth that generates hyperplasia in the lungs of asthmatic patients.

3.6 | Measure of angiogenesis and fibrosis markers in BSMC after exosome addition

We therefore hypothesized that exosomes could affect gene expression in factors linked to angiogenesis and fibrotic processes. *CCR3* and *VEGFA* gene expression was augmented in BSMC cultured with exosomes from asthmatics at 24 hours (2.37 ± 1.10 -fold and 2.30 ± 0.93 -fold, respectively; $P < .05$, Figure 6). We also measured *TGFβ1*, *MMP9*, *TNF* and *FGF2*; nevertheless, we did not find any changes in these genes at any time-point (Figure S3).

At all time-points assayed, *CCR3* and *VEGFA* expression showed similar levels between cells cultured with exosomes from healthy subjects and negative controls to both genes ($P > .05$). When we compared gene expression between both conditions (ie exosomes from asthmatics and healthy individuals), significant differences were observed in *CCR3* and *VEGFA* expression at 24 hours (2.37 ± 1.10 -fold vs 1.21 ± 1.07 -fold, $P < .05$ and 2.30 ± 0.93 -fold vs 0.98 ± 0.17 -fold $P < .001$, respectively), indicating that the observed effects are due to asthma.

3.7 | Exosome uptake by structural lung cells

Exosomes began to internalize inside both types of cells at 15–20 minutes, reaching maximum fluorescence at 60 minutes after exosome addition. Figure 7 shows representative confocal microscopy images of exosome uptake by SAEC (Figure 7A) and BSMC (Figure 7B). We observed that both types of exosomes (healthy and asthmatics) were internalized in the same manner, and were grouped into the perinuclear region, but did not enter the nucleus. Also, it may be that SAEC or BSMC incubated with exosomes from asthmatic patients have a higher fluorescence signal (green channel) than cells incubated with exosomes of eosinophils from healthy subjects (SAEC: 6.48 ± 3.75 vs 3.27 ± 1.08 arbitrary units of fluorescence intensity, respectively, $P < .05$; BSMC: 3.52 ± 2.58 vs 1.36 ± 1.05 , arbitrary units of fluorescence intensity, respectively, $P < .05$). These results mean that the uptake is greater with eosinophil-derived exosomes from asthmatics than uptake in exosomes from healthy donors. To discard the possibility of an unspecific signal, cells were incubated with the fluorescent probe without exosomes. In this case, we did not observe any fluorescent signal, neither in the cytoplasm nor in other cell locations (Figure 7).

4 | DISCUSSION

In this study, we have demonstrated that asthmatic eosinophil-derived exosomes can influence the function of structural lung cells,

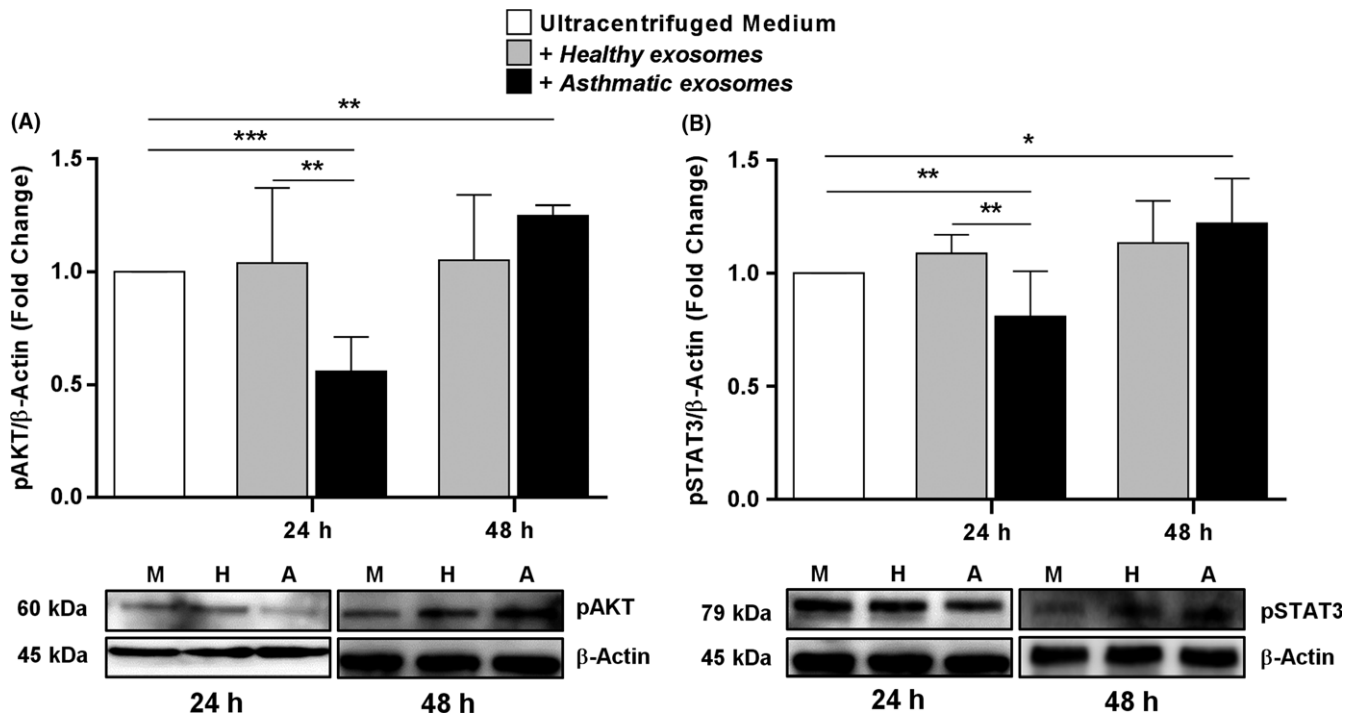


FIGURE 4 Involvement of exosomes from asthmatics in phosphorylation of AKT and STAT3 factors in SAEC. Exosomes can modify the phosphorylation pattern of both signalling factors. A, At 24 h, exosomes produced a significant decrease in phosphorylated levels of AKT in comparison with negative control ($n = 6$) and with cells cultured with exosomes from healthy individuals ($n = 6$). However, at 48 h a significant increase in the amount of pAKT in respect to negative control was observed ($n = 4$). B, The same effect occurred in pSTAT3 levels. At 24 h, a significant decrease in pSTAT3 was observed in respect to control cells ($n = 12$), and cells cultured with exosomes from healthy individuals ($n = 5$), whereas at 48 h exosomes produce only a statistically significant increase compared to negative control ($n = 6$). Amounts of both signalling factors were normalized to β -actin. M: protein extract from SAEC cultured without exosomes; H: protein lysates from SAEC cultured with exosomes from healthy donors; A: protein extract from SAEC cultured with exosomes from asthmatic patients. * $P < .05$; ** $P < .01$; *** $P < .001$

modifying several processes linked to these cells (SAEC and BSMC) and changing the expression profile of several pro-inflammatory molecules. We previously demonstrated that exosomes from eosinophils of asthmatic patients act as an extracellular functional unit, playing an important role in several processes associated with asthma pathogenesis. Moreover, exosomes released by eosinophils of asthmatic patients appear to exert relevant effects over eosinophils, the main effector cells in asthma.¹⁴ In this study, therefore, we determined whether eosinophil-derived exosomes modulate structural lung cell functions and contribute to the remodelling process as seen in asthma.

Our results show that the presence of exosomes increases the apoptosis rate in SAEC, and delays the repair of established epithelial damage. Moreover, exosomes of eosinophils from asthmatic patients increase the gene expression of *TNF*, *CCL26* and *POSTN*, thereby prolonging inflammation status. In addition to serving as a protective barrier, the airway epithelium orchestrates the host inflammatory response.^{7,9} As in impaired epithelial repair, dysfunctional wound healing has been shown to trigger the pathognomonic features of asthma. Wound repair is therefore essential to maintain the functionality of the epithelium as an immunological barrier against external agents. The altered gene expression induced by exosomes of

asthmatic eosinophils seen in our study is similar to that present in the lung epithelium of asthmatic patients.^{19,20}

Exosomes have a pro-inflammatory effect on structural cells.^{21,22} This injury unleashes the expression of several cytokines by the airway epithelium, such as CCL26/eotaxin-3 and periostin.

Periostin, a 90 kDa protein, is believed to interact with multiple integrins²³ and is involved in different processes such as epithelial-mesenchymal transformation and fibrosis.²⁴ Several groups have confirmed higher expression of this protein in asthmatic airways. A relation has been shown between levels of periostin and subepithelial fibrosis mechanisms such as the reduction in airway distensibility.^{21,25} Moreover, serum periostin levels have been correlated with eosinophilic inflammation,²⁶ with a rapid worsening of pulmonary function,²⁷ and with a type-2 immunity asthma.²⁸ The increase in periostin expression in SAEC induced by the presence of exosomes from eosinophils of asthmatic patients could act as a cell-binding matrix, specifically facilitating eosinophil tissue infiltration and creating positive inflammatory feedback.

Several studies have demonstrated the role of JAK/STAT and PI3K/AKT signalling pathways in the features of asthma. These pathways are implicated in the regulation of cell survival, migration, angiogenesis and motility, as well as wound repair of epithelial

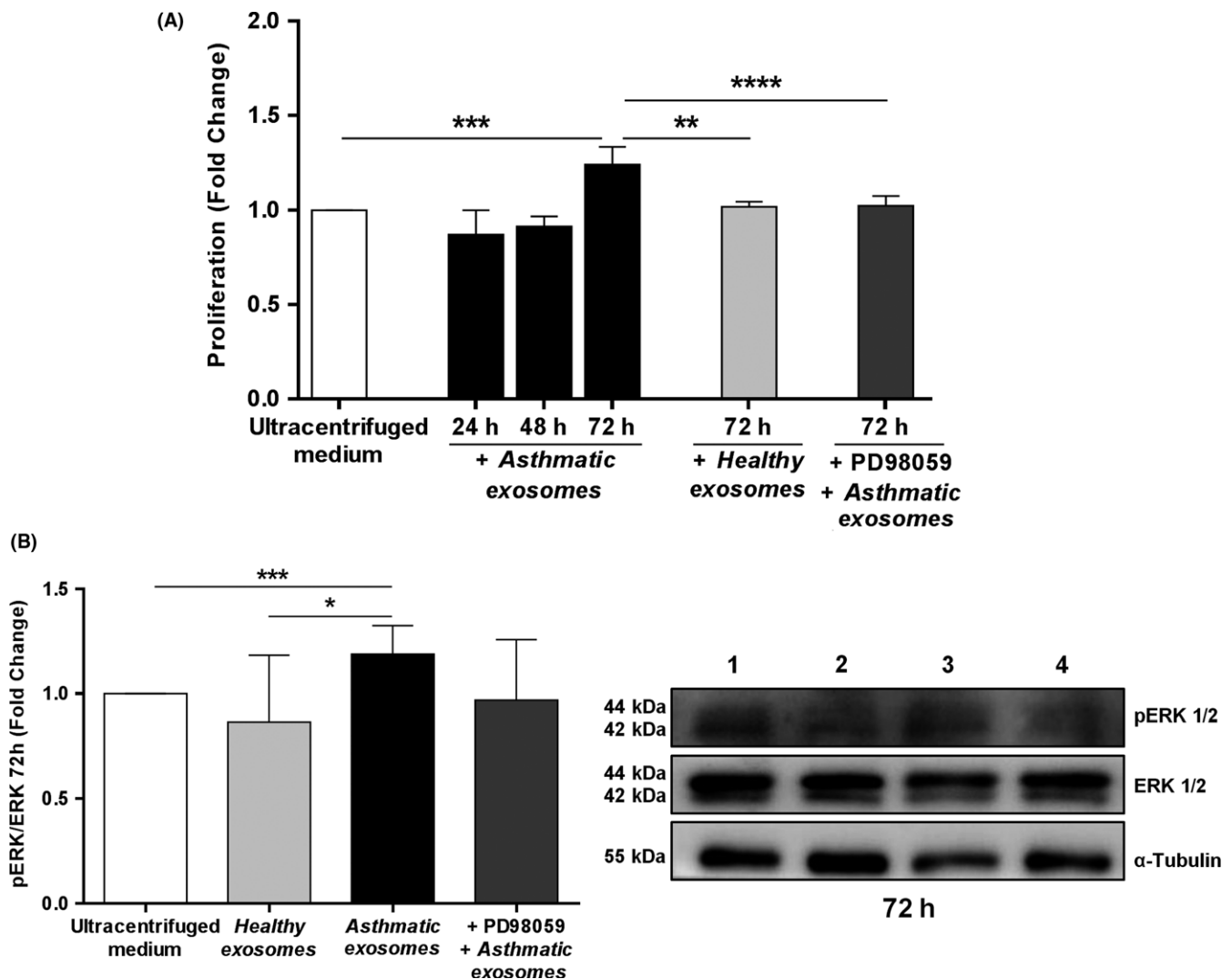


FIGURE 5 Exosomes of eosinophils from asthmatic patients induce proliferation in BSMC. A, Time course effect of eosinophil-derived exosomes from asthma and healthy group on BSMC proliferation. Figures are plotted as mean $\pm$ SD ($n = 7$) of fold change relative to BSMC cultured without exosomes. Exosomes from asthmatic patients vs control cells (ultracentrifuged medium). Exosomes from asthmatic patients vs cells cultured with exosomes from healthy individuals. Inhibition of pERK vs exosomes from asthmatics, $n = 9$. Healthy-derived exosomes addition did not show any significant effect in respect to control cells ($n = 8$). B, Analysis of pERK1/2 protein levels in BSMC. Representative pERK1/2, ERK1/2 and α -tubulin Western blot of BSMC cultured with and without exosomes from asthmatic and healthy eosinophils for 72 h. Exosomes from asthmatics vs control cells (ultracentrifuged medium), $n = 11$. Exosomes from asthmatics vs cells cultured with exosomes from healthy subjects, $n = 7$. Densitometric quantification is expressed as fold change relative to negative control (BSMC cultured without exosomes). Lane 1: control (protein extract from BSMC cultured without exosomes). Lane 2: protein extract from BSMC cultured with exosomes from a healthy subject. Lane 3: protein extract cultured with exosomes from the asthmatic patient. Lane 4: protein extract from BSMC previously cultured with PD98059 (pERK inhibitor) before asthmatic exosome addition. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$

cells.^{29,30} In our study, exosomes significantly decreased the phosphorylation of STAT3 and AKT factors in SAEC at 24 hours. At the same time, apoptosis levels are increased in these cells and wound repair is slowed when exosomes are present. One hypothesis arising from this is that an association exists between low levels of pSTAT3 and pAKT, and that an increase in apoptosis and a loss of motility and migration take place in SAEC. STAT3 has an anti-apoptotic effect, activating mechanisms that block apoptosis³¹ and promoting epithelial regeneration after injury.³² Also, AKT has been described as a critical factor for the regulation and blockade of apoptosis and

promotion of cell survival,³³ because epithelial wound repair is dependent on pAKT levels. In this context, Wang et al²⁹ have demonstrated that inhibitors of AKT phosphorylation attenuate the epithelium reparation. However, at 48 hours, a significant increase has been observed in both pAKT and pSTAT3 levels.

Remodelling is a key feature in the pathogenesis of chronic asthma. ASM cell proliferation contributes to this process. It is a main aetiological factor in the airway narrowing that accompanies this disease.³⁴ We have proved that exosomes from eosinophils of asthmatic patients actively participate in bronchial muscle hyperplasia.

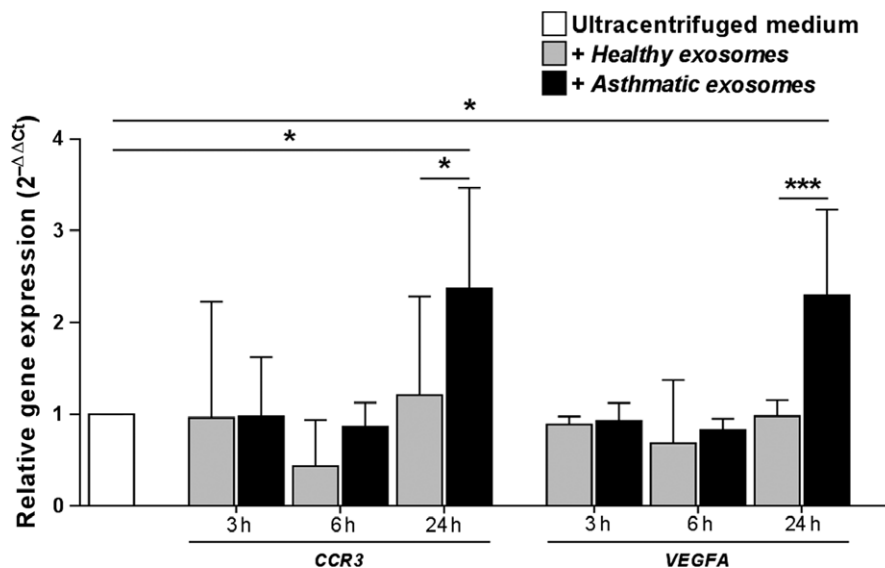


FIGURE 6 Effect of eosinophil-derived exosomes on the expression of cytokines by human BSMC. mRNAs were quantified using a qPCR. Results are expressed as mean $\pm$ SD for 6 independent experiments performed in triplicate. * $P < .05$, *** $P < .001$

Thus, exosome-cultured BSMC show a non-significant reduction in growth at 24 hours and 48 hours compared to controls; nevertheless, at 72 hours, we observed elevated proliferation compared to controls, reaching statistical significance. This increase is linked to a rise in MAPK expression, specifically pERK1/2. The MAPK family is implicated in growth factor signalling, proliferation and apoptosis.³⁵ The existence of dual pathways (ERK and PI3-kinase) regulating ASM cell proliferation in healthy samples was previously established.³⁶ However, if both pathways are involved in the promotion of the abnormal proliferation process in asthmatic status, the process is not totally defined. Our results have shown a clear connection between the increased proliferation of non-asthmatic BSMC stimulated with exosomes from eosinophils of asthmatic patients and ERK1/2 activation. Moreover, these results are supported by ERK1/2 inhibition assay, where proliferation at 72 hours is decreased when BSMC are incubated with the specific inhibitor of pERK, PD98059. Similar results showing that growth of muscle cells is linked to ERK1/2 phosphorylation have been published.³⁷

When we evaluated the expression of certain genes in ASM cells incubated with exosomes from asthmatics at different time-points, we observed that CCR3 and VEGFA gene expression is increased at 24 hours. CCR3 activation is an important pathway that triggers the proliferation process through the MAPK cascade. This receptor can be used by several eotaxins, and even other chemokines such as RANTES, MCP-2 or MIP-1 δ , which transduces and activates a number of pathways.³⁸ Thus, the increased BSMC proliferation that we observed when culturing these cells with exosomes from asthmatic patients can occur through CCR3, which induces ERK phosphorylation and generates abnormal proliferation. These same findings are supported by several previous studies.^{39,40} In addition, the higher expression of VEGFA observed could also be implicated in this mechanism. A raised level of this factor is a feature of asthma associated with bronchial wall remodelling.⁴¹

These observations are consistent with our previous results suggesting that exosomes from eosinophils of asthmatic patients could

be pro-inflammatory vehicles, acting over BSMC to trigger the MAPK cascade, specifically ERK1/2 phosphorylation. This process leads to increased proliferation, which ultimately results in muscle hyperplasia.

Neither of these cell types showed any alteration in their function when incubated with exosomes from healthy subjects. A possible explanation is that quantitative protein composition differs between healthy and asthmatic exosomes, even where exosome qualitative composition is similar between them.¹⁴ The greater uptake of exosomes of eosinophils from asthmatics patients likely drives the effects observed (Figure 7).

Finally, cell-exosome interaction has been studied, although the exact mechanisms are still unclear. The 2 most likely choices of exosome uptake are endocytosis⁴² and fusion with the plasma membrane. In our study, exosomes were internalized towards the cytoplasm by SAEC and BSMC, and fluorescence was located inside the cell in the perinuclear region. Therefore, the most probable mechanism is endocytosis. Internalization and localization in the perinuclear region of exosomes by epithelial and smooth muscle cells, and originated by another cell type (such as dendritic cells or neutrophils), have been observed in other studies, indicating that this is probably necessary to exert their effects.^{43,44}

In our study on asthma, exosomes from eosinophils seem to help trigger an inflammatory response in the airways, increasing the activation and recruitment of eosinophils from peripheral blood to the lung. There, these leucocytes exert their pro-inflammatory activities on structural, airway epithelial and smooth muscle cells, likely through exosome release. In addition, a feedback loop could be established by expression of different factors and receptors, such as periostin and CCR3. This facilitates and promotes the expansion of the inflammatory response, increasing inflammatory cellular infiltration and muscle and epithelial remodelling, which leads to chronic inflammation status. Therefore, exosomes from eosinophils of asthmatic patients may play an essential role in this airway inflammation perpetuation, exerting multiple functions on several levels.

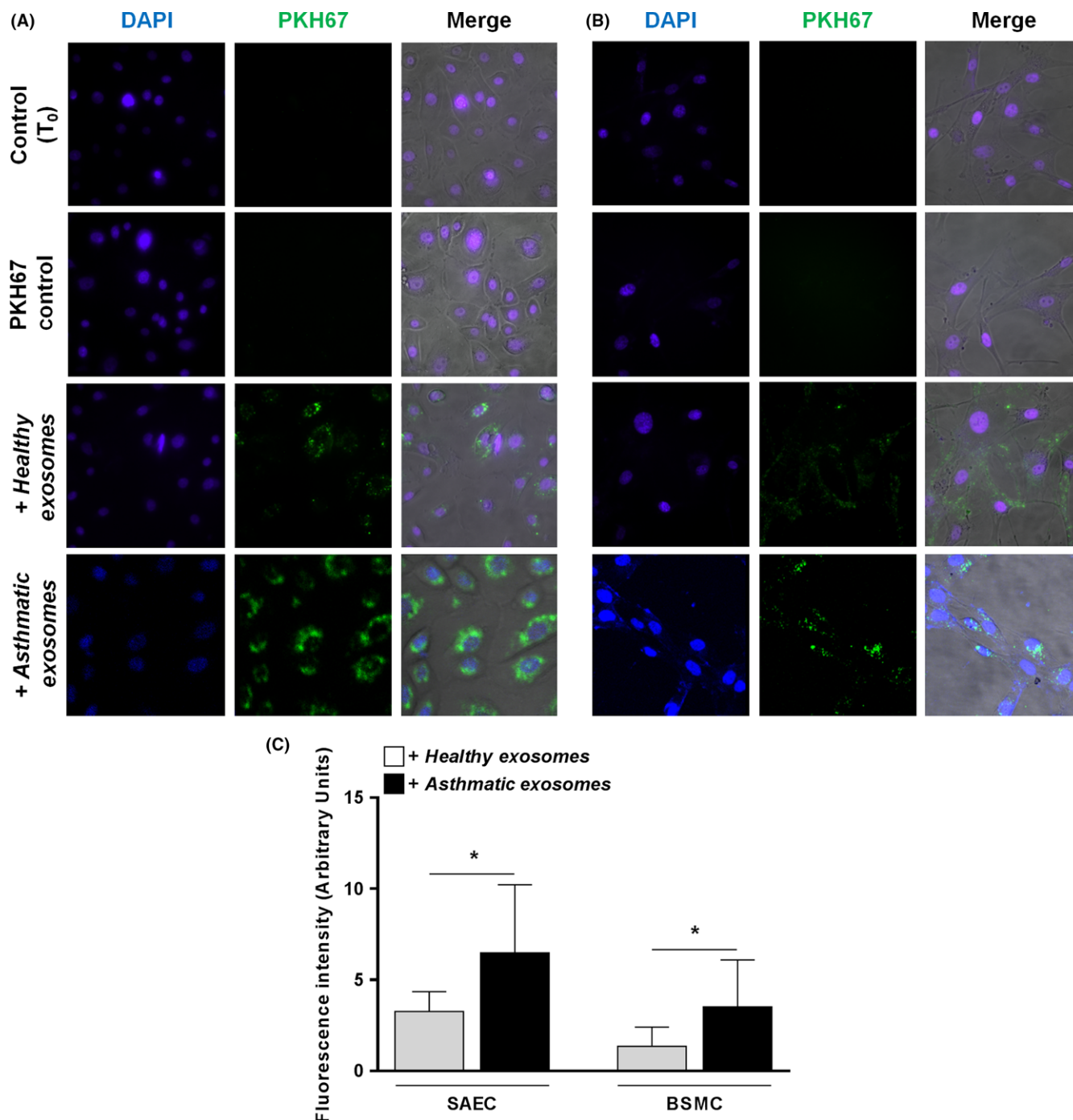


FIGURE 7 BSMC and SAE cellular uptake of eosinophil-derived exosomes from asthma and healthy groups. A and B. Figure shows representative images of exosomes uptake performed by confocal microscopy at maximum fluorescence intensity at 60 min. SAE (left panel) and BSMC (right panel) were incubated in the presence of exosomes of eosinophils labelled with PKH67 fluorescent probe (green) from asthmatic and healthy subjects for 3 h. Nuclei were labelled with DAPI (purple/blue). Images were taken from an intracellular z plan with confocal microscopy, and the stronger green signals represent exosomes. C, Quantification of fluorescence intensity produced by internalization of labelled exosomes in 8 different fields. * $P < .05$

In summary, this work adds to the knowledge base regarding the capacity of eosinophils to contribute to the inflammatory response, as exosomes derived from eosinophils act as functional units contributing to the persistence and exacerbation of asthma. Clarifying this phenomenon will be of great importance if exosomes are

considered a viable future therapy approach. Within the field of asthma research, there is currently no specific approach concerning how to use them; in cancer research, on the other hand, some papers have demonstrated that exosomes from tumorigenic cells are organotropism biomarkers that could be employed in personalized

medicine.^{11,45,46} For asthma, exosomes could be used as a therapy target via exosome-cell interaction blockade. In this manner, the effect of exosomes over recipient cells could be minimized or voided. Our results appear to open a new line of research in the treatment of this respiratory disease.

ACKNOWLEDGEMENTS

This study was supported by Fondo de Investigación Sanitaria—FIS [PI15/00803]; CIBER de Enfermedades Respiratorias (CIBERES), a Carlos III Institute of Health Initiative; Conchita Rábago Foundation (FCR); and FEDER funds.

The authors recognize Oliver Shaw, English editor of IIS-FJD, for his revision and editing in English. The authors thank Carla Mazzeo and Manuel Izquierdo for technical support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

V. del Pozo  <http://orcid.org/0000-0001-6228-1969>

REFERENCES

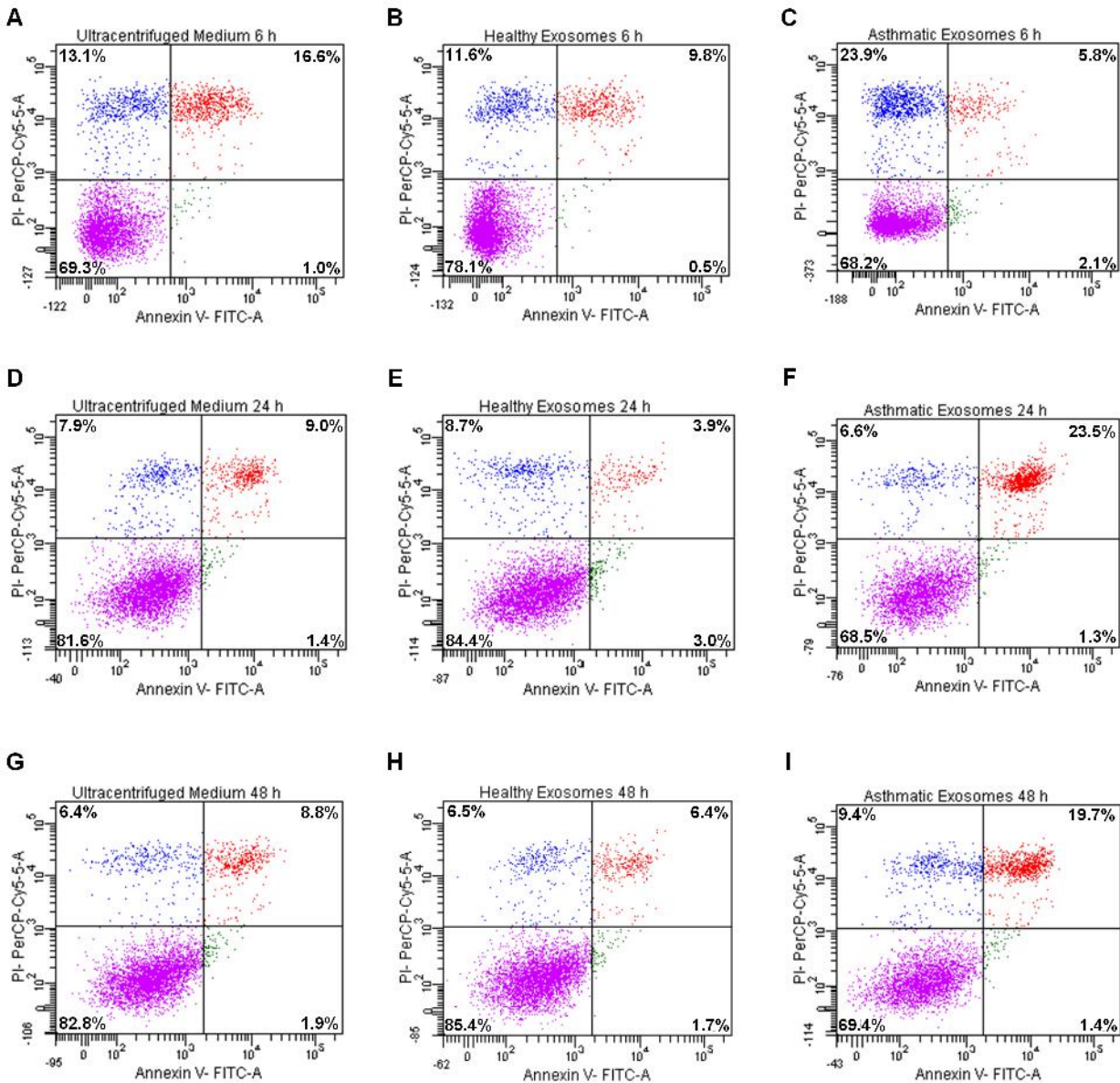
- Reddel HK, Bateman ED, Becker A, et al. A summary of the new GINA strategy: a roadmap to asthma control. *Eur Respir J*. 2015;46:622-639.
- Kudo M, Ishigatsubo Y, Aoki I. Pathology of asthma. *Front Microbiol*. 2013;4:263.
- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med*. 2000;161:1720-1745.
- Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol*. 2001;28:233-236.
- Kaur D, Hollins F, Saunders R, et al. Airway smooth muscle proliferation and survival is not modulated by mast cells. *Clin Exp Allergy*. 2010;40:279-288.
- Knox AJ, Corbett L, Stocks J, Holland E, Zhu YM, Pang L. Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism. *FASEB J*. 2001;15:2480-2488.
- Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. *J Allergy Clin Immunol*. 2011;128:451-462.
- Roth M, Johnson PR, Borger P, et al. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med*. 2004;351:560-574.
- Cho JY. Recent advances in mechanisms and treatments of airway remodeling in asthma: a message from the bench side to the clinic. *Korean J Intern Med*. 2011;26:367-383.
- Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta*. 2012;1820:940-948.
- Hoshino A, Costa-Silva B, Shen TL, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015;527:329-335.
- Torregrosa Paredes P, Esser J, Admyre C, et al. Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. *Allergy*. 2012;67:911-919.
- Mazzeo C, Cañas JA, Zafra MP, et al. Exosome secretion by eosinophils: a possible role in asthma pathogenesis. *J Allergy Clin Immunol*. 2015;135:1603-1613.
- Cañas JA, Sastre B, Mazzeo C, et al. Exosomes from eosinophils autoregulate and promote eosinophil functions. *J Leukoc Biol*. 2017;101:1191-1199.
- American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. *Am Rev Respir Dis*. 1987;136:225-244.
- López E, Zafra MP, Sastre B, et al. Suppressors of cytokine signaling 3 expression in eosinophils: regulation by PGE₂ and Th2 cytokines. *Clin Dev Immunol*. 2011;2011:917015.
- Chomczynski P, Mackey K. Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques*. 1995;19:942-945.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.
- Coleman JM, Naik C, Holguin F, et al. Epithelial eotaxin-2 and eotaxin-3 expression: relation to asthma severity, luminal eosinophilia and age at onset. *Thorax*. 2012;67:1061-1066.
- Parulekar AD, Atik MA, Hanania NA. Periostin, a novel biomarker of TH2-driven asthma. *Curr Opin Pulm Med*. 2014;20:60-65.
- Sidhu SS, Yuan S, Innes AL, et al. Roles of epithelial cell-derived periostin in TGF-beta activation, collagen production, and collagen gel elasticity in asthma. *Proc Natl Acad Sci U S A*. 2010;107:14170-14175.
- Larose MC, Chakir J, Archambault AS, et al. Correlation between CCL26 production by human bronchial epithelial cells and airway eosinophils: involvement in patients with severe eosinophilic asthma. *J Allergy Clin Immunol*. 2015;136:904-913.
- Johansson MW, Annis DS, Mosher DF. $\alpha_M\beta_2$ integrin-mediated adhesion and motility of IL-5-stimulated eosinophils on periostin. *Am J Respir Cell Mol Biol*. 2013;48:503-510.
- Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY, Chang DD. Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. *Cancer Res*. 2002;62:5358-5364.
- Takayama G, Arima K, Kanaji T, et al. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol*. 2006;118:98-104.
- Jia G, Erickson RW, Choy DF, et al. Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma (BOBCAT) Study Group. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol*. 2012;130:e10.
- Kanemitsu Y, Matsumoto H, Izuhara K, et al. Increased periostin associates with greater airflow limitation in patients receiving inhaled corticosteroids. *J Allergy Clin Immunol*. 2013;132:e3.
- Johansson MW, Evans MD, Crisafi GM, Holweg CTJ, Matthews JG, Jarjour NN. Serum periostin is associated with type 2 immunity in severe asthma. *J Allergy Clin Immunol*. 2016;137:e2.
- Wang WC, Kuo CY, Tzang BS, Chen HM, Kao SH. IL-6 augmented motility of airway epithelial cell BEAS-2B via Akt/GSK-3 β signaling pathway. *J Cell Biochem*. 2012;113:3567-3575.
- Kida H, Mucenski ML, Thitoff AR, et al. GP130-STAT3 regulates epithelial cell migration and is required for repair of the bronchiolar epithelium. *Am J Pathol*. 2008;172:1542-1554.
- Matsuzaki Y, Xu Y, Ikegami M, et al. Stat3 is required for cytoprotection of the respiratory epithelium during adenoviral infection. *J Immunol*. 2006;177:527-537.

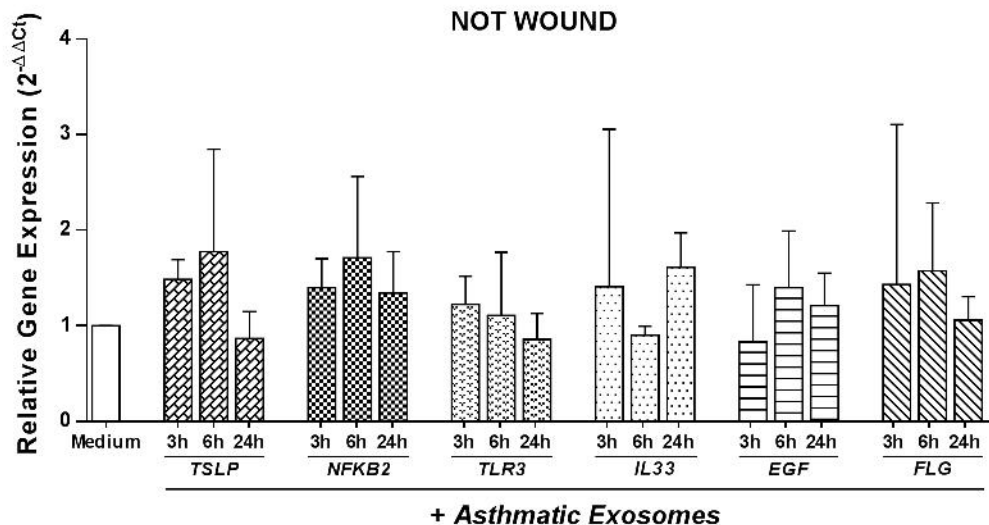
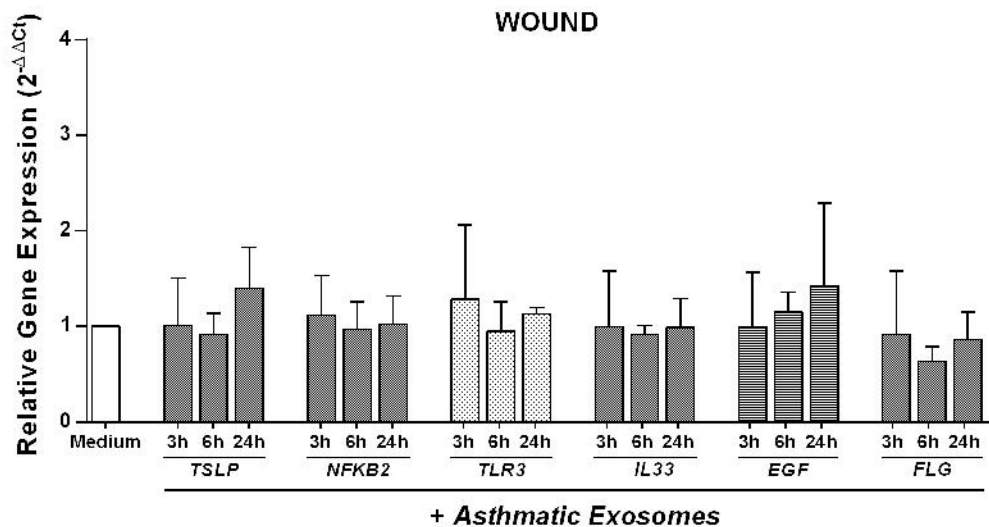
32. Tadokoro T, Wang Y, Barak LS, Bai Y, Randell SH, Hogan BL. IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells. *Proc Natl Acad Sci U S A*. 2014;111:E3641-E3649.
33. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. 2005;9:59-71.
34. Johnson PR, Roth M, Tamm M, et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med*. 2001;164:474-477.
35. Redhu NS, Shan L, Al-Subait D, et al. IgE induces proliferation in human airway smooth muscle cells: role of MAPK and STAT3 pathways. *Allergy Asthma Clin Immunol*. 2013;9:41.
36. Lee JH, Johnson PR, Roth M, Hunt NH, Black JL. ERK activation and mitogenesis in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 2001;280:L1019-L1029.
37. Burgess JK, Lee JH, Ge Q, et al. Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma. *J Cell Physiol*. 2008;216:673-679.
38. Errahali YJ, Taka E, Abonyo BO, Heiman AS. CCL26-targeted siRNA treatment of alveolar type II cells decreases expression of CCR3-binding chemokines and reduces eosinophil migration: implications in asthma therapy. *J Interferon Cytokine Res*. 2009;29:227-239.
39. Halwani R, Al-Abri J, Beland M, et al. CC and CXC chemokines induce airway smooth muscle proliferation and survival. *J Immunol*. 2011;186:4156-4163.
40. Markwick LJ, Clements D, Roberts ME, Ceresa CC, Knox AJ, Johnson SR. CCR3 induced-p42/44 MAPK activation protects against staurosporine induced-DNA fragmentation but not apoptosis in airway smooth muscle cells. *Clin Exp Allergy*. 2012;42:1040-1050.
41. Chetta A, Zanini A, Foresi A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma. *Clin Exp Allergy*. 2005;35:1437-1442.
42. Morelli AE, Larregina AT, Shufesky WJ, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood*. 2004;104:3257-3266.
43. Obregon C, Rothen-Rutishauser B, Gerber P, Gehr P, Nicod LP. Active uptake of dendritic cell-derived exovesicles by epithelial cells induces the release of inflammatory mediators through a TNF- α -mediated pathway. *Am J Pathol*. 2009;175:696-705.
44. Vargas A, Roux-Dalvai F, Droit A, Lavoie J-P. Neutrophil-derived exosomes: a new mechanism contributing to airway smooth muscle remodeling. *Am J Respir Cell Mol Biol*. 2016;55:450-461.
45. Liu Y, Cao X. Organotropic metastasis: role of tumor exosomes. *Cell Res*. 2016;26:149-150.
46. Weidle UH, Birzele F, Kollmorgen G, R ger R. The multiple roles of exosomes in metastasis. *Cancer Genomics Proteomics*. 2017;14:1-15.

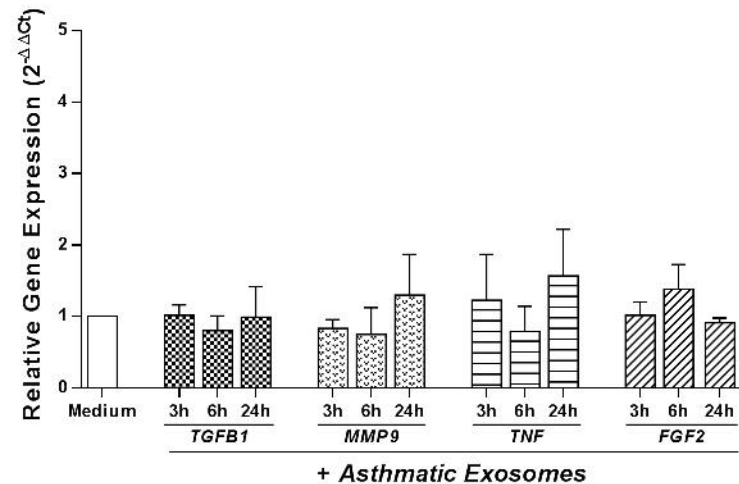
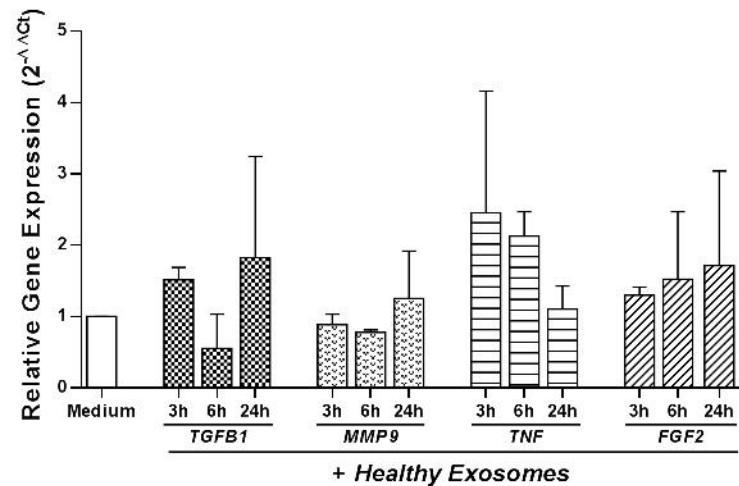
SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Ca as JA, Sastre B, Rodrigo-Mu oz JM, et al. Eosinophil-derived exosomes contribute to asthma remodelling by activating structural lung cells. *Clin Exp Allergy*. 2018;48:1173-1185. <https://doi.org/10.1111/cea.13122>



A**B**

A**B**

SUPPLEMENTARY INFORMATION

Supplementary Figure 1. Percentage of apoptotic cells by flow cytometry.

Representative flow cytometry dot plots from the different conditions assayed. **A-C.** Representative dot plots at 6 h of cells cultured with ultracentrifuged medium (A), SAEC incubated with exosomes from healthy subjects (B) and SAEC incubated with exosomes from asthmatics (C). **D-F.** Representative dot plots at 24 h of cells cultured with ultracentrifuged medium (D), SAEC incubated with exosomes from healthy subjects (E) and SAEC incubated with exosomes from asthmatic donors (F). **G-I.** Representative dot plots at 48 h of cells cultured with ultracentrifuged medium (G), SAEC incubated with exosomes from healthy individuals (H) and SAEC incubated with exosomes from asthmatic patients (I).

Supplementary Figure 2. Incubation of SAEC with exosomes from asthmatic patients did not induce changes in expression of several pro-inflammatory genes.

Relative gene expression of several genes on SAEC incubated with exosomes from asthmatic patients in both cases: without or with wound in cell monolayer. **A.** Six genes were evaluated in SAEC cultured without wound ($n = 5$). **B.** The same genes were assayed in SAEC cultures with wound ($n = 5$). In both cases no significant differences were observed ($P > 0.05$) Statistics were performed with Mann-Whitney test for non-parametric data and unpaired t test for parametric data. All experiments were performed in triplicate.

Supplementary Figure 3. Eosinophil-derived exosomes from healthy and asthmatic donors did not modify expression of several genes on BSMC. These cells were incubated during different times with exosomes from healthy and asthmatic individuals,

and then relative gene expression was evaluated ($n = 5$). Statistics were performed with Mann-Whitney test for non-parametric data and unpaired t test for parametric data. All experiments were performed in triplicate.

Supplementary Video 1: Time-lapse video of wound healing assay. SAEC were cultured until 100% confluence. A wound was made in the cell monolayer with the tip of a 200- μ L pipette and incubated with exosomes from healthy and asthmatics, or without them. The left panel shows wound healing in SAEC without exosomes. The central panel shows the damage repair in SAEC incubated with exosomes from healthy subjects. The right panel displays SAEC wound repair with exosomes from eosinophils of asthmatic patients. Video has been made with the xvid mpeg-4 codec.