Exosome secretion by eosinophils: A possible role in asthma pathogenesis

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Background: Eosinophils secrete several granules that are involved in the propagation of inflammatory responses in patients with pathologies such as asthma.
Objective: We hypothesized that some of these granules are exosomes, which, when transferred to the recipient cells, could modulate asthma progression.
Methods: Eosinophils were purified from peripheral blood and cultured with or without IFN-γ or eotaxin. Multivesicular bodies (MVBs) in eosinophils were studied by using fluorescence microscopy, transmission electron microscopy (TEM), and flow cytometry. Exosome secretion was measured and exosome characterization was performed with TEM, Western blotting, and NanoSight analysis.
Results: Generation of MVBs in eosinophils was confirmed by using fluorescence microscopy and flow cytometry and corroborated by means of TEM. Having established that eosinophils contain MVBs, our aim was to demonstrate that eosinophils secrete exosomes. To do this, we purified exosomes from culture medium of eosinophils and characterized them. Using Western blot analysis, we demonstrated that eosinophils secreted exosomes and that the discharge of exosomes to extracellular media increases after IFN-γ stimulation. We measured exosome size and quantified exosome production from healthy and asthmatic subjects using nanotracking analysis. We found that exosome production was augmented in asthmatic patients.
Conclusion: Our findings are the first to demonstrate that eosinophils contain functional MVBs and secrete exosomes and that their secretion is increased in asthmatic patients. Thus exosomes might play an important role in the progression of asthma and eventually be considered a biomarker. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Asthma, eosinophils, exosomes, multivesicular bodies, biomarker, IFN-γ, endosomes, lysobisphosphatidic acid, CD63, secretion*

Abbreviations used

ECP:	Eosinophil cationic protein
EPO:	Eeosinophil peroxidase
GFP:	Green fluorescent protein
ILV:	Intraluminal vesicle
LBPA:	Lysobisphosphatidic acid
MBP:	Major basic protein
MFI:	Mean fluorescence intensity
MVB:	Multivesicular body
PKC:	Protein kinase C
TEM:	Transmission electron microscopy
WB:	Western blot

Eosinophils are recruited in large numbers at the site of allergic inflammation, parasitic infections, and other immune responses.[1] These immune leukocytes are not only destructive effectors cells but also active players in immune modulation, tissue repair processes, and normal organ development in both health and disease.[2-4]

Eosinophils have the capacity to synthesize, store within intracellular granules, and immediately secrete a diverse repertoire of cytokines, chemokines, and other important mediators. Secretory granules or vesicles of eosinophils are storage organelles in which molecules from the trans-Golgi network are concentrated and packaged. When appropriately stimulated, secretory granules are selectively mobilized to the plasma membrane. Once there, they undergo complex fusion events to secrete their content to the extracellular space.[5,6]

From the trans-Golgi network, the endosomal system controls the uptake and processing of various macromolecules. Most cells have vesicular organelles, which are interconnected and consist basically of primary endosomes, late endosomes, and lysosomes.[7] $CD63^+$ late endosomes generate intraluminal vesicles (ILVs) rich in lysobisphosphatidic acid (LBPA) into their lumen in a process called maturation. Through this process, late endosomes are transformed into mature $CD63^+/LBPA^+$ multivesicular bodies (MVBs).[8] MVBs not only fuse to lysosomes to degrade their intraluminal cargo but also fuse to the plasma membrane to release ILVs into the extracellular space as exosomes.[7,9] Exosomes are small vesicles that contain bioactive lipids, nucleic acid, and proteins, which are delivered to different locations in the body. One of the most important functions of exosomes appears to be intercellular communication.[10] Exosomes are secreted, constitutively and on stimulation, by different types of cells, and thus their composition differs depending on their cellular origin. They also have specific molecules related to their biogenesis, which allow their characterization (exocarta.org/exosome_markers). Exosomes have been defined by their size, density, and expression of specific biomarkers (eg, tetraspanins).[11] Exosomes can be found in abundance in body fluids,

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such as blood, saliva, urine, breast milk, and bronchoalveolar lavage fluid.[12-17] Thus the exosomal pathway constitutes a mechanism for local and systemic intercellular transfer of information.[10] Because of their abundance and unique composition, they might represent ideal biomarkers for diagnosis and prognosis of a wide variety of diseases.[18]

Asthma is a common chronic inflammatory disorder characterized by variable airflow obstruction in association with airway hyperresponsiveness. The release of potent inflammatory mediators and the remodeling of the airway wall promote airway dysfunction.[19] The late/chronic phase is mainly attributed to the effects of infiltrating eosinophils and their derivatives.[20]

Exosomes released from both immune and structural cells in the lung[21,22] have recently been shown to play an important role in local regulation of asthma pathology.[23] Moreover, exosomes from asthmatic patients differ in quantity, composition, and function compared with those from healthy subjects,[24] which demonstrates the implication of exosomes in regulation of asthma pathology. Therefore it is important to discern which cell types are involved in exosome production.[25]

Recent studies have found vesicles produced by eosinophils in the extracellular space functioning autonomously and independently outside of eosinophils.[26,27] It is possible that these vesicles from eosinophils participate in the exacerbation of asthma. Our study was based on the theory that some of those vesicles could be eosinophil exosomes with a significant implication in asthma progression.

METHODS

Sample collection and eosinophil isolation

Samples of peripheral blood (50 mL) from asthmatic patients (n = 20) and healthy subjects (n = 10) were obtained voluntarily and after obtaining written informed consent. Patients with asthma had a consistent history of the disease and objective evidence of asthma (as defined by the American Thoracic Society)[28] for at least 6 months. These patients either showed a greater than 12% improvement in FEV_1 10 minutes after administration of 500 μg of inhaled terbutaline or had methacholine airway hyperresponsiveness (PC_{20} methacholine <16 mg/mL). Asthmatic patients had mild persistent disease[29] and were clinically stable. None had a history of respiratory tract infections for at least the 6-week period preceding the study. We included both atopic and nonatopic patients in the asthmatic group because no differences in the parameters assessed for both sets of patients had been observed previously. For patients who were receiving inhaled corticosteroids, the drugs were withdrawn for at least 2 weeks before blood extraction. No patient was receiving oral corticosteroids (for at least 6 months before the study), leukotriene receptor antagonists, aspirin, or any other COX inhibitor.

Eosinophils were purified from the peripheral blood of healthy control subject and patient donors by using a 2-step procedure, as previously described.[30] Briefly, the polymorphonuclear cell fraction was obtained by using Ficoll gradient centrifugation. The second step involved removal of residual cells from the polymorphonuclear cell fraction. To purify eosinophil, $CD2^+$, $CD3^+$, $CD14^+$, $CD16^+$, $CD19^+$, $CD20^+$, $CD36^+$, $CD56^+$, $CD123^+$, and glycophorin A–positive cells were discarded by using a magnetic bead separation technique, as described in the manufacturer's instructions (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada). Eosinophil viability and purity were routinely greater than 98%. Purified eosinophils were suspended in RMPI-1640 medium (Sigma-Aldrich, St Louis, Mo) supplemented with 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, and 10% (vol/vol) FBS (Lonza, Basel, Switzerland), which had been stripped of bovine exosomes by means of ultracentrifugation. The culture medium was also supplemented with a cocktail of IL-5 (5 ng/mL) and GM-CSF (10 ng/mL). The culture cells were maintained at 37°C in a 5% CO_2 atmosphere for 24 hours.

Transfection assays and expression vectors

Eosinophils were transiently transfected with 20 μg of the pEFGFP-C1bosCD63[31] plasmid in a total volume of 650 μL. The culture was maintained at 37°C in a 5% CO_2 atmosphere for 24 hours. Routine transfection efficiency was around 75%.

Time-lapse assays

At 24 hours after transfection, eosinophils were attached to glass-bottom 35-mm culture dishes (MatTek, Ashland, Mass) coated with fibronectin. Once the cells were placed in the microscope chamber, eosinophils were stimulated with 20 ng/mL IFN-γ (R&D Systems, Minneapolis, Minn) in culture medium and maintained in an automatic OKO Lab System (Okolab SRL, Naples, Italy) that controls CO_2 levels, temperature, and humidity.[32] Subsequently, epifluorescent images were taken with a Nikon Eclipse TE2000S microscope equipped with a DS-Qi1Mc digital camera and a Plan Apo VC 60 NA 1.4 objective. Time-lapse analysis was performed in living cells by using NIS-AR software (Nikon Instruments, Melville, NY). Time-lapse images were homogeneously taken every minute by using a band pass–specific filter for green fluorescent protein (GFP) to avoid fluorescence quenching with a 1-second exposure time. Sequence image acquisition was coordinated with an automatic fluorescence shutter to avoid fluorescence bleaching. Epifluorescent image improvement was achieved by using Huygens deconvolution software (SVI, Hilversum, The Netherlands).

Fluorescence and confocal laser-scanning microscopy

After 24 hours of culture, the samples were fixed and intracellularly labeled, as described previously,[32] with anti-tetraspanin CD63 (Immunostep S.L., Salamanca, Spain) primary antibody, a reporter of endosomal vesicles, and anti-LBPA primary antibody (Echelon Biosciences, Salt Lake City, Utah), which is a specific marker of MVBs,[33] and an appropriate secondary fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ) and Alexa Fluor 647–conjugated antibody (Invitrogen, Madison, Wis). Epifluorescent images were taken with the same microscope and digital camera described for time-lapse analysis. For quantification, digital images were analyzed with NIS-AR software (Nikon Instruments). The experimental significance of the results obtained at the single-cell level was achieved by analyzing a minimum number of 50 cells per treatment from different microscope fields. Results were expressed as the mean of sum intensity LBPA fluorescence/area of the cells. Confocal microscopy was performed with a Leica TCS SP5 scan head mounted on a Leica microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) and a 63 NA 1.4 Plan Apo objective. Images of the cells were acquired from a 0.7-μm optical section, and no labeling was observed when using the secondary antibody alone. Merged images were performed, taking the z axis maximum intensity projection (which takes the intensity values of individual pixels in all sections and collapses them into a single illuminated image). ImageJ software was used for analysis (National Institutes of Health, Bethesda, Md; http://rsb.info.nih.gov/ij),[34] and colocalization indexes were analyzed with the JACoP plugin for ImageJ software.

Induction of exosome secretion and inhibition assay

For the induction of exosome secretion, 2×10^6 eosinophils per well were treated with or without 20 ng/mL IFN-γ (R&D Systems) for 10 minutes and with or without 100 ng/mL eotaxin (R&D Systems) for 1 hour. Inhibition assays were performed by adding 2 μmol/L protein kinase C (PKC) inhibitor (Gö6985) for 20 minutes before IFN-γ treatment.

Exosome purification

Exosomes produced by an equal number of cells per each experimental condition were isolated from the cell-culture supernatants, as previously

described.[35] By using these standard protocols, culture supernatants of the cells were centrifuged at low speed in sequential steps and then clarified to eliminate cells and cell debris. The exosomes were finally recovered by means of ultracentrifugation (100,000*g* for 16 hours), as previously described.[31]

SDS-PAGE and Western blot analysis

Cells and recovered exosomes were lysed with RIPA buffer containing protease inhibitors, and the proteins were separated by means of SDS-PAGE (10%) and transferred to Hybond-P ECL membranes (GE Healthcare, Buckinghamshire, United Kingdom). Membranes were labeled with specific molecules related to exosome biogenesis, which allow their characterization, such as anti-CD63 (Calbiochem, San Diego, Calif), anti-CD9 (Immunostep, Salamanca, Spain), and anti-Alix (Cell Signaling Technology, Danvers, Mass). CD63, CD9, and Alix are specific exosome proteins included in the list of the 25 proteins most often identified in exosomes (exocarta.org/exosome_markers). For CD63 detection, proteins were separated under nonreducing conditions, as described previously.[36] Membranes were also labeled with specific molecules related to eosinophils, such as anti–eosinophil peroxidase (EPO; Chemicon International, Temecula, Calif), anti–major basic protein (MBP; Chemicon International), and anti–eosinophil cationic protein (ECP; Santa Cruz Biotechnology, Dallas, Tex). After incubation with the appropriate primary antibody, the blots were developed with horseradish peroxidase–conjugated secondary antibodies by using enhanced chemiluminescence reagents and according to standard protocols. In experiments involving the isolation of exosomes and Western blot (WB) analysis, each lane of the blot contained the total exosomes recovered in culture medium from the same number of eosinophils either untreated or treated with IFN-γ in the presence or absence of PKC inhibitor.

Nanoparticle Tracking Analysis

Size distribution and quantification of exosome preparations were analyzed by measuring the rate of Brownian motion with a NanoSight LM10 system (NanoSight, Wiltshire, United Kingdom), which is equipped with fast video capture and particle-tracking software.[37,38] Purified exosomes from asthmatic patients and healthy subjects were diluted in 500 μL of 1× PBS/5 mmol/L EDTA and disaggregated by using a syringe and needle (1 mL 29-gauge × ½). After this procedure, the sample was injected in a NanoSight sample cubicle. The size of eosinophil exosomes was obtained by determining the mean ± SD size distribution. The mean of the number of particles acquired per milliliter was compared between asthmatic patients and healthy subjects.

Transmission and immune electron microscopy

Eosinophils in suspension were fixed in a 10-fold dilution of a fix solution (1% paraformaldehyde, 1.5% glutaraldehyde, and 0.025% $CaCl_2$ in 0.1 mol/L sodium cacodylate buffer, pH 7.4) at 20°C. After fixation for 1 hour at room temperature, cells were washed and resuspended in cold 0.1 mol/L sodium cacodylate buffer (pH 7.4) before processing for electron microscopy.[39,40] Nickel grids containing sections of cells were immunolabeled on a 50-μL drop, as described previously.[40] Immunolabeling was carried out with anti-LBPA antibody (Echelon Biosciences) and 5-nm gold-conjugated anti-mouse secondary antibody (Sigma-Aldrich) for detection with electron microscopy.

Exosomes suspended in 1× PBS/5 mmol/L EDTA were dropped on nickel grids and fixed with 2% paraformaldehyde and incubated either with anti-CD63 or anti-Alix antibodies, and then a secondary 5-nm gold-conjugated secondary antibody was used for detection with electron microscopy. The primary antibodies and gold-conjugated secondary antibody were diluted in 0.1% BAS in 1× PBS. The samples were treated with 2.5% glutaraldehyde and contrasted by adding 2% uranyl acetate. The preparations were examined with a JEOL JEM1010 transmission electron microscope (JEOL USA, Peabody, Mass) at 80 Kv for cells and 100 Kv for exosomes.

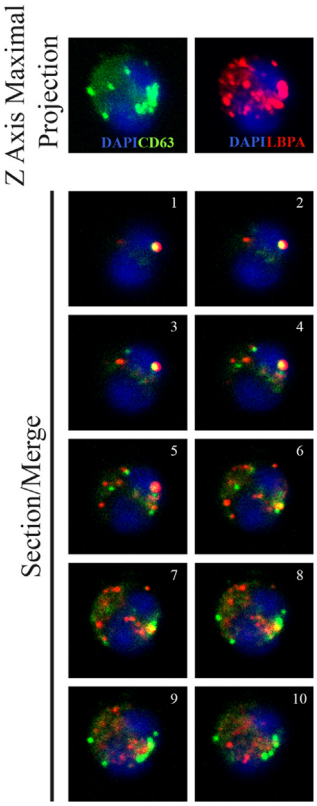

FIG 1. Detection of MVBs in eosinophils by using confocal microscopy. The *upper panels* show the z axis maximum-intensity projection of sections of LBPA- and CD63-labeled eosinophils, and the *bottom panels* show a stack of merged images, with coincident labeling appearing in yellow and indicating the presence of MVBs. Analysis with the JACoP plugin for ImageJ software indicated that in section 7 colocalization is partial, with different intensities (Pearson coefficient, $r = 0.62$). In this experiment 6 human donors and 20 cells per donor were analyzed.

Flow cytometry

Eosinophils were fixed and intracellularly labeled, as described previously,[32] with anti-CD63 (Immunostep) and anti-LBPA (Echelon Biosciences) primary antibodies and appropriate phycoerythrin- or fluorescein isothiocyanate–conjugated secondary antibodies (BD Biosciences). A minimum of 1×10^5 cells were analyzed for FL-1 or FL-2 fluorescence by using the BD FACSCanto II (BD Biosciences). Experimental significance of the results was achieved by analyzing the LBPA and CD63 mean fluorescence intensity (MFI) from eosinophils with or without IFN-γ treatment.

Data analysis

Results were compared by using ANOVA and unpaired *t* tests with the Welch correction for nonparametric and parametric data, respectively. Normality was analyzed by using the Kolmogorov-Smirnov test. A difference was considered significant at a *P* value of .05 or less. Statistical analyses were performed with GraphPad InStat3 software (GraphPad Software, San Diego, Calif).

RESULTS

Detection and characterization of MVBs on eosinophils

To determine whether eosinophils have the capacity to generate exosome precursors, we first examined the presence of MVBs by labeling 2×10^6 cells with anti-CD63 (green) and anti-LBPA

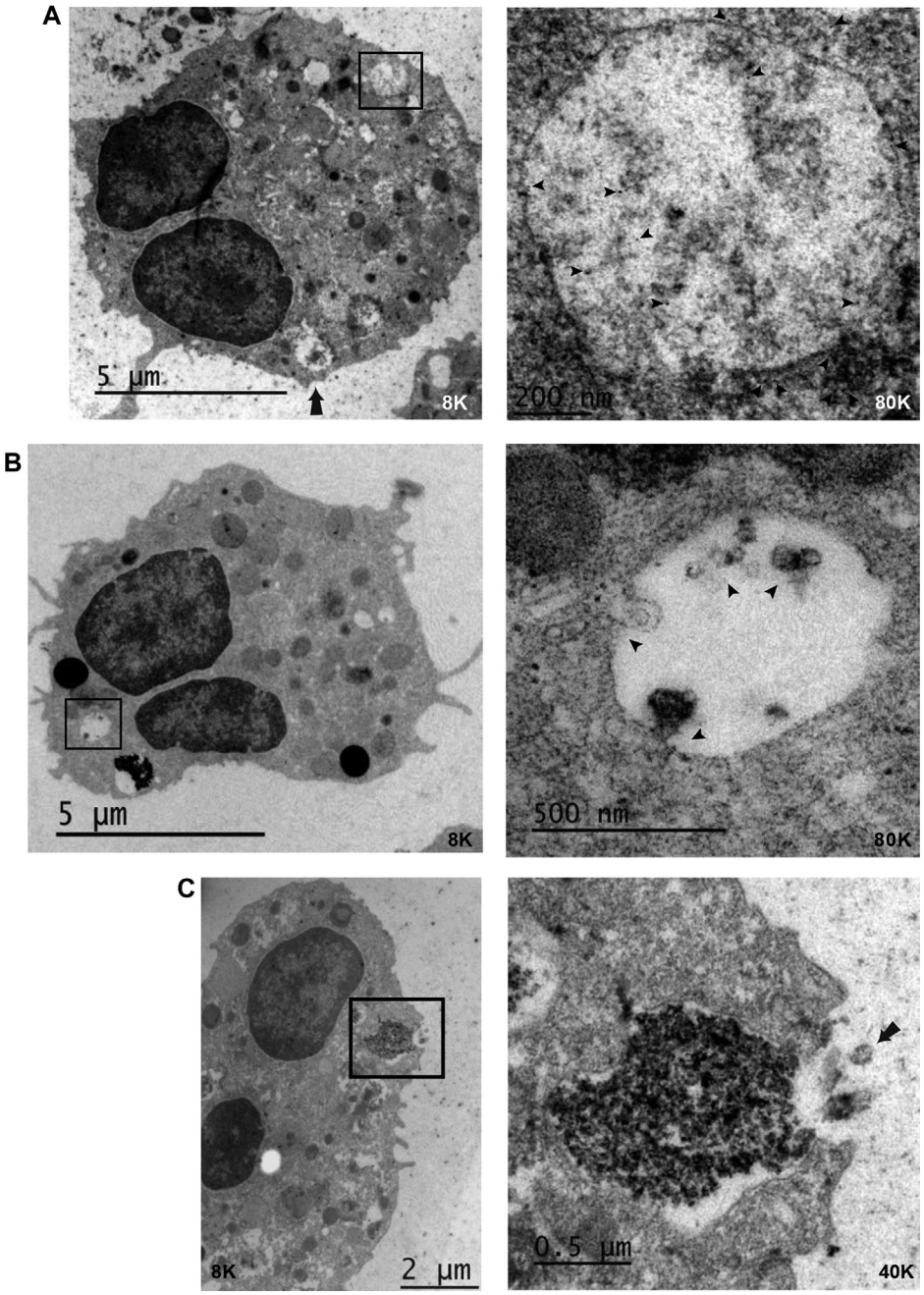

FIG 2. Detection of MVBs in eosinophils by using TEM. **A,** 8,000× magnification of eosinophils. *Arrows* indicate the presence of MVBs. The *rectangle* indicates 1 selected MVB (80,000× magnification). *Arrowheads* show the presence of LBPA. **B,** 8,000× magnification of eosinophils. The *rectangle* indicates the presence of 1 mature MVB (80,000× magnification). *Arrowheads* show the process of inward budding of MVBs and also some ILVs. **C,** 8,000× magnification of 1 eosinophil. The *rectangle* indicates the fusion of 1 MVB with the plasma membrane and concomitant secretion on its content. 40,000× magnification of the MVB fusion is shown. The *arrow* indicates the presence of one extracellular vesicle with the features of an exosome. This image is of a representative eosinophil of 44 analyzed from 10 different human donors.

(red) in 6 human donors. Analysis of 20 independent eosinophils by using confocal microscopy showed that immunoreactivity against CD63 and LBPA colocalizes in some granules, as highlighted by the yellow color observed in the merged image (Fig 1). This fact indicates that some of the $CD63^+$ (green) endosomes correspond to mature MVBs because they also are $LBPA^+$ (red). The indicators of colocalization, in section 7, point out that the colocalization is partial, with different intensities (Pearson coefficient, $r = 0.65$; Van Steensel cross-correlation coefficient, CCFmin = 0.060/CCFmax = 0.62; Li intensity correlation coefficient, ICQ = 0.40; Costes randomization, $r = 0.62$).

To verify these data, we analyzed eosinophils (n = 44) by using transmission electron microscopy (TEM) from 10 different

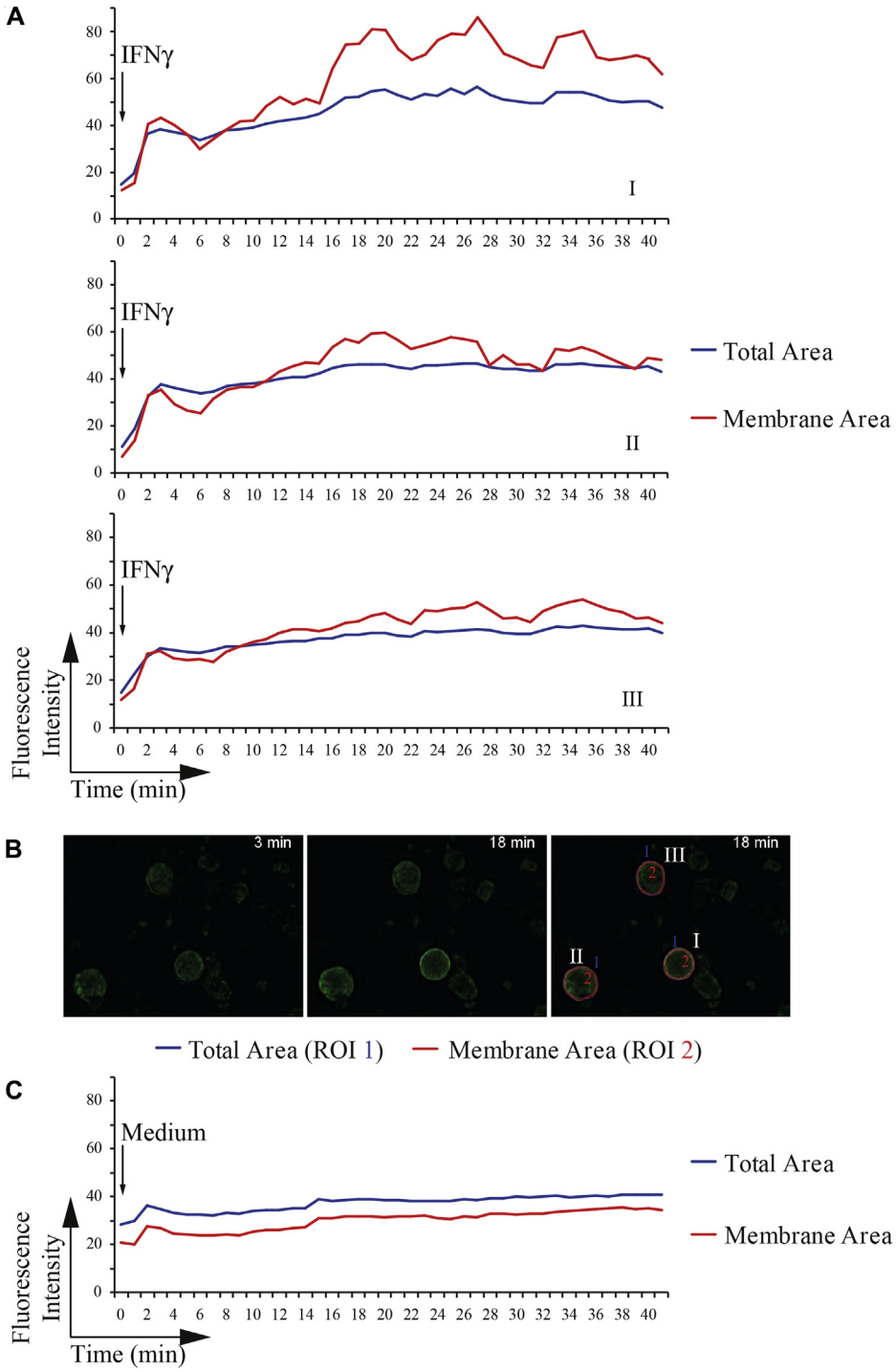

FIG 3. Time-course study of IFN-γ induction degranulation of eosinophil vesicles expressing GFP-CD63. Time-lapse acquisition (1 frame per minute; exposure, 1 second) and analysis were performed in transfected eosinophils (75% efficiency). **A** and **B,** Results of viable eosinophils (n = 3; *I*, *II*, and *III*) stimulated with IFN-γ. **C,** Unstimulated eosinophils were also analyzed (n = 7). The figure shows a representation of the intensity of fluorescence with respect to time in 2 defined regions of interest (*ROI 1*, total cell area; *ROI 2*, plasma membrane area) from Video E1. A switch in the fluorescence intensity from cytoplasm to the plasma membrane was observed after 8 minutes in stimulated eosinophils.

asthmatic patients. LBPA$^+$ ILVs contained in vesicles displaying the structural characteristics of MVBs were observed (Fig 2, *A*). In Fig 2, *B*, a representative example of both inward budding of the limiting membrane of MVBs and ILVs in eosinophils is shown (Fig 2, *B*). The images also show the fusion of MVBs with the plasma membrane and secretion of microvesicles compatible

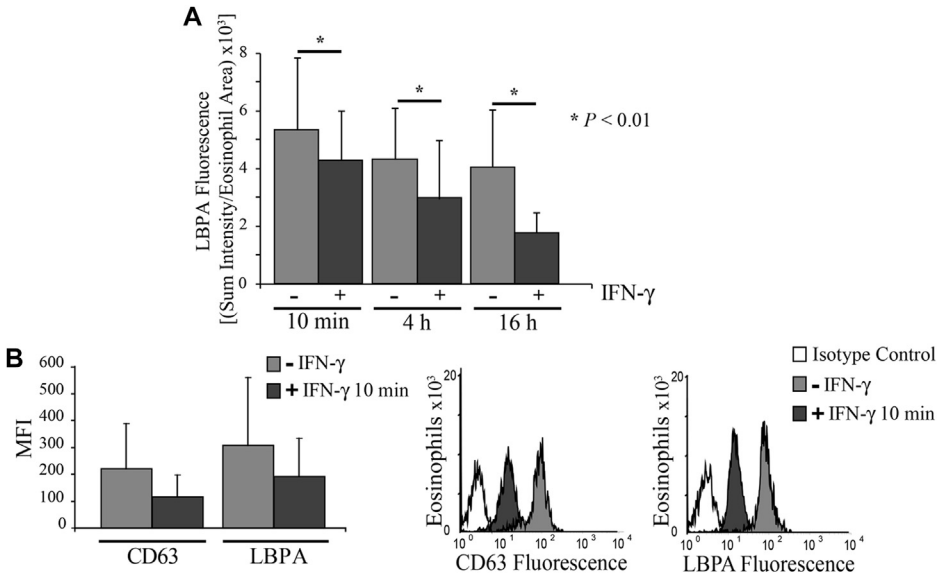

FIG 4. MVB traffic after IFN-γ stimulation of eosinophils. **A,** Eosinophils (2×10^6) from 2 different donors were cultured with or without IFN-γ. At different times, cells were collected, fixed, permeabilized, and labeled with LBPA–Alexa Fluor 647. Epifluorescent images were taken, and the LBPA fluorescence intensity was measured in 50 cells in each condition. The sum intensity of LBPA fluorescence (mean ± SD per cell area) was obtained at 10 minutes, 4 hours, and 16 hours after stimulation and compared with that seen in cells without stimuli. The difference was significant at every time point of treatment ($P < .05$). **B,** Eosinophils (2×10^6) from 3 different donors were cultured with or without IFN-γ. After 10 minutes, cells were collected, fixed, permeabilized, and labeled with CD63 and LBPA. The fluorescence intensity was measured by using flow cytometry; typically, 50,000 to 100,000 total events were acquired. Data represent MFI ± SD (n = 3). A representative histogram showing $CD63^+$ and $LBPA^+$ cells and isotype labeling is displayed.

with the size and shape of exosomes (Fig 2, *C*). Taken together, these data indicate that the eosinophils contain MVBs distributed in their cytoplasm.

IFN-γ and granule mobilization

To produce intracellular movement of granules, we used IFN-γ because it has been demonstrated to produce a selective and rapid evacuation of stored primary granules.[41-43] To study the ability of IFN-γ to mobilize eosinophil granules to the plasma membrane and induce their degranulation, we performed time-lapse microscopy. The behavior of GFP-$CD63^+$ eosinophil vesicles after IFN-γ stimulation was visualized in transfected living cells (75% efficiency). The kinetic changes of CD63-GFP levels, in 3 eosinophils (which appeared in the field of time-lapse acquisition) stimulated with IFN-γ and 7 unstimulated eosinophils (as controls), were tracking. The time-lapse assay showed an enhancement of GFP-CD63 fluorescence at the plasma membrane between 8 and 10 minutes after IFN-γ stimulation (Fig 3, *A*, and see Video E1 in this article's Online Repository at www.jacionline.org). The plasma membrane increased its fluorescence because of the presence of GFP-CD63, indicating that fusion of GFP-$CD63^+$ vesicles occurred only after stimulation (Fig 3, *A* and *B*). This increase of membrane fluorescence was not observed in unstimulated cells (Fig 3, *C*). We represented the time course of fluorescence changes in 2 defined regions of interest (ROI 1, total cell area; ROI 2, plasma membrane) obtained from Video E1 (Fig 3, *B*). A switch in the fluorescence intensity from the cytoplasm to the plasma membrane was observed after 8 minutes.

LBPA fluorescence intensity was also measured on eosinophils with and without IFN-γ treatment at different times by means of analysis with epifluorescence microscopy (a total of 50 cells were analyzed in each condition from 2 donors). The sum intensity of LBPA fluorescence (mean ± SD/cell area) was obtained at different times after stimulation and compared with that seen in cells without stimuli at each time. As can be seen in Fig 4, *A*, a progressive decrease of LBPA fluorescence in the cytoplasm of eosinophils was detected at 10 minutes, 4 hours, and 16 hours after induction when compared with that seen in control cells without stimulation. The difference in LBPA fluorescence between stimulated and nonstimulated eosinophils was significant at every time point of IFN-γ treatment ($P < .05$).

We also measured the total MFI of LBPA and CD63 with or without induction by using flow cytometry (typically 50,000 to 100,000 total cells were analyzed). A decreasing trend in MFI was observed after 10 minutes of IFN-γ treatment (Fig 4, *B*). Data represent MFI ± SD values (n = 3).

Characterization of eosinophil exosomes

Having established that eosinophils contain the precursors of exosomes and these MVBs fuse to the plasma membrane on stimulation, our aim was to demonstrate that eosinophil MVBs are productive and secrete exosomes. Thus we purified exosomes from the culture medium of eosinophils obtained from donors and characterized them by using different techniques.

WB. WB analysis was performed on exosome lysates from the culture supernatants of 2×10^6 eosinophils cultured with or without IFN-γ or eotaxin. Membranes were labeled with specific

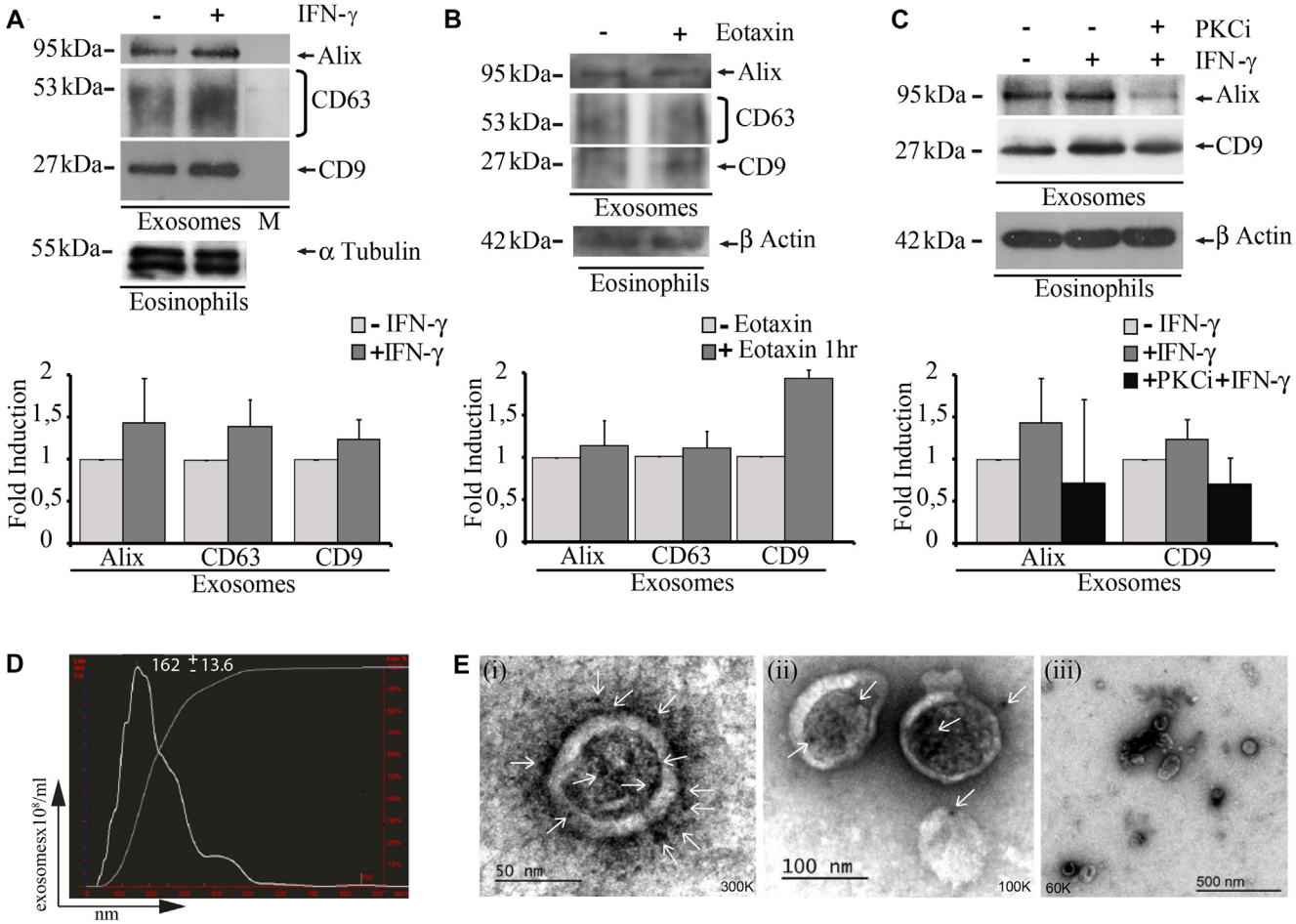

FIG 5. Characterization of eosinophil-derived exosomes. **A,** WB analysis from exosomes was achieved by using antibodies against Alix, CD9, and CD63 on IFN-γ induction. α-Tubulin was used as a loading control in eosinophils to show that exosomes come from the same number of eosinophils. *M*, Medium. Exosome-depleted culture medium was used as a control for the cell-free condition. **B,** WB analysis from exosomes was achieved by using antibodies against Alix, CD9, and CD63 on eotaxin induction. α-Tubulin was used as a loading control in eosinophils. **C,** Alix and CD9 WB analysis was performed from exosomes on IFN-γ induction in the presence *(+)* or absence *(−)* of PKC inhibitor (*PKCi*; 5 μmol/L). β-Actin was used as a loading control in eosinophils. Fig 5, *A-C*, are representative examples of several WBs, all displaying similar results. Bands were quantified by densitometry, and histograms with means ± SDs (n = 3) are shown. **D,** Exosomes were measured by using NanoSight LM10 in the supernatant from cultures of 2×10^6 eosinophils (n = 18). The histogram represents particle size distribution (exosome $\times 10^8$/mL vs size in nanometers). **E,** TEM of eosinophil exosomes showing their ultrastructure. Vesicles display the characteristic cup-shape morphology of exosomes. *Arrowheads* indicate positive immunogold labeling: Alix *(i)* or CD63 *(ii)*. This image is a representative of 22 exosomes tested from 6 different donors. *(iii)*, Representative image of exosome preparation from eosinophils under low magnification.

molecules related to exosome biogenesis, which makes their characterization possible (exocarta.org/exosome_markers). As depicted in Fig 5, *A* and *B*, a strong labeling of Alix (95 kDa), CD63 (53 kDa), and CD9 (25 kDa) proteins was detected in isolated exosomes from the supernatant of equal numbers of eosinophils. Unlike IFN-γ, eotaxin does not appear to increase numbers of $Alix^+$ and $CD63^+$ exosomes, whereas CD9 levels might increase.

Vesicle secretion is a mechanism triggered by an increase in intracellular calcium concentration, followed by PKC activation.[31,44-46] To elucidate whether the secretion of exosomes by eosinophils is an actively regulated process that not only can be induced but also can be arrested, we set up experiments with Gö6985, a PKC inhibitor. Preincubation with Gö6985 before culture with IFN-γ resulted in a partial inhibition in exosome secretion (66%) measured as a reduction in CD9 expression (Fig 5, *C*), and thus inhibition of PKC abrogates IFN-γ–induced stimulation.

Nanoparticle Tracking Analysis. We have also measured the size distribution and concentration of eosinophil exosomes by using a NanoSight LM10 instrument. Exosomes were isolated from culture medium and purified by means of differential ultracentrifugation, and after disaggregation in PBS/EDTA, they were injected in the NanoSight LM10 chamber (see Video E2 in this article's Online Repository at www.jacionline.org). All independent samples of exosomes (n = 18) registered a

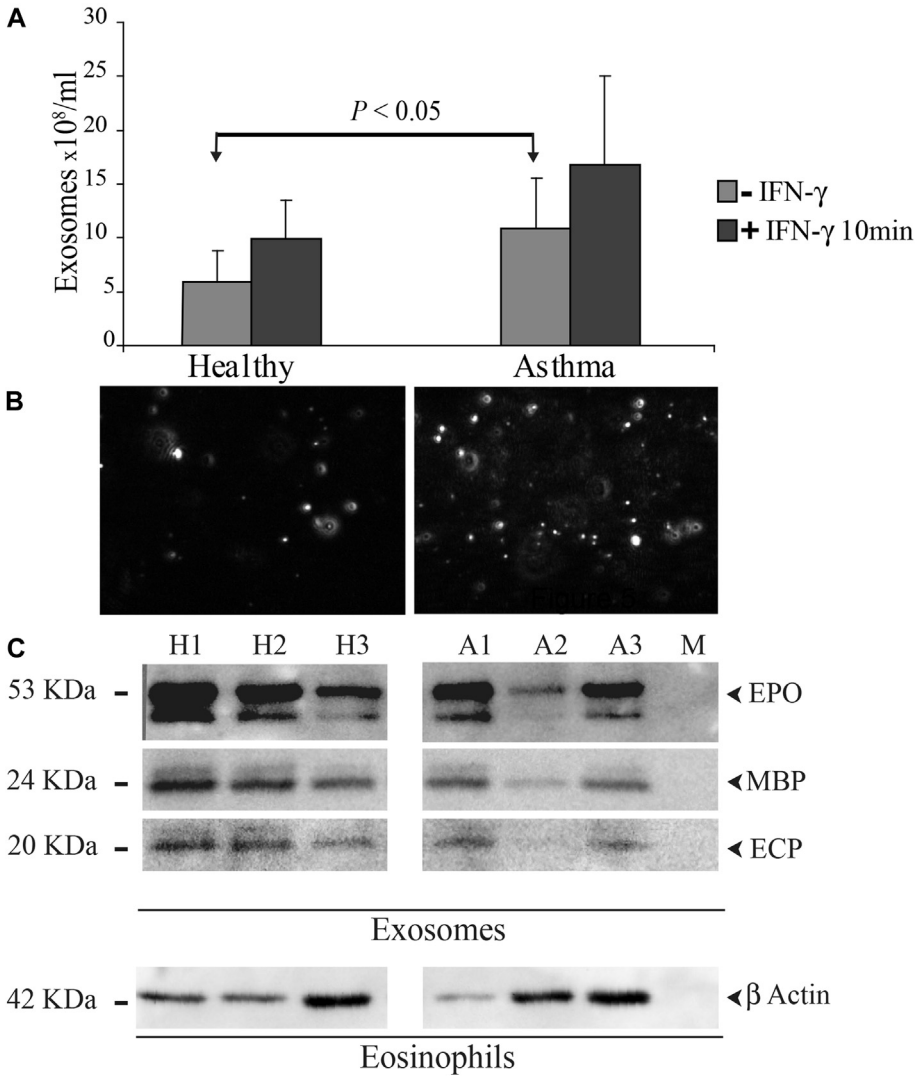

FIG 6. Eosinophil exosome secretion from healthy subjects and asthmatic patients. Exosomes isolated from the culture medium of 2×10^6 eosinophils from healthy subjects and asthmatic patients induced or not with IFN-γ for 10 minutes were quantified by using NanoSight LM10 analysis. **A,** The bar graph represents means ± SDs of exosome concentrations from 7 asthmatic patients and 5 healthy subjects. **B,** Screen shots of video from NanoSight LM10 of eosinophil exosome light scatter showing the difference in secretion between healthy subjects and asthmatic patients. **C,** WB of exosome lysates from healthy subjects *(H)* and asthmatic patients *(A)* was performed with EPO, MBP, and ECP antibodies. *M,* Medium. Exosome-depleted culture medium was used as a control for cell-free condition. β-Actin was assayed as a loading control to show that exosomes come from the same number of eosinophils.

similar size distribution. The mean size distribution was 162 ± 13.6 nm (Fig 5, *D*), which corresponds with the exosome size.

TEM. Eosinophil exosomes were also characterized by using TEM. Isolated exosomes were disaggregated and fixed before immunogold labeling with primary antibodies directed against characteristic exosomal proteins (Alix and CD63). The electron microscopic images showed vesicles with the characteristic cup-shape morphology of exosomes. Arrowheads over membranous vesicles indicate positive label to CD63 or Alix proteins (Fig 5, *E*). A TEM image obtained with low magnification is shown in Fig 5, *E*.

All these results confirm the presence of exosomes in eosinophil culture supernatants.

Eosinophil exosomes detected in asthmatic patients

Taking into account the relevance of eosinophils in asthmatic patients, we decided to investigate whether there is any difference between exosomes secreted by eosinophils from healthy subjects and asthmatic patients. Eosinophils purified from different blood samples of 5 healthy subjects and 7 asthmatic patients were treated with IFN-γ or left untreated to induce exosome secretion. The exosomes purified from the eosinophil culture supernatants from healthy subjects and asthmatic patients were quantified by using the NanoSight LM10. As can be observed in Fig 6, higher production of exosomes was detected in unstimulated eosinophils from asthmatic patients than in those from healthy subjects ($10.86 \pm 4.72 \times 10^8$ vs $5.92 \pm 2.91 \times 10^8$, $P < .05$). Moreover,

the stimulation of eosinophil secretion by IFN-γ produced a similar increase in exosome secretion (1.5-fold for asthmatic patients vs 1.6-fold for healthy subjects, Fig 6). There were no differences in the size distribution of exosomes from healthy subjects and asthmatic patients (data not shown).

We also investigated whether eosinophil-derived exosomes contained molecules that are potentially relevant to human asthma, and therefore we performed WB against eosinophil granule proteins, such as EPO, MBP, and ECP, in healthy subjects and asthmatic patients. As seen in Fig 6, *C*, eosinophil-derived exosomes from healthy subjects and asthmatic patients contain EPO, MBP, and ECP. We did not observe differences in EPO, MBP, and ECP expression between healthy subjects and asthmatic patients.

DISCUSSION

In this article, for the first time, we have shown that eosinophils contain MVBs (exosome precursors) and are able to secrete exosomes to extracellular media. Moreover, this response was enhanced by IFN-γ treatment. The study of the presence of MVBs among eosinophil granules has been a fundamental step toward continuing investigation of eosinophil exosomes. The analysis of $LBPA^+$ granules in eosinophils was essential to this study because LBPA is an important factor that controls exosome biogenesis.[33] LBPA provides the negative curvature to the limiting membrane of MVBs during the inward budding to form ILVs. After docking and fusion of MVBs with the plasma membrane, the ILVs they contain are secreted as exosomes to the extracellular space. By using LBPA as a marker of MVBs, we have been able not only to detect MVBs inside the eosinophils by using confocal microscopy but also to confirm their presence through analysis of their ultrastructure with TEM. The immunogold particle appeared to be localized to the organelle matrix; presumably, this label is localized to the ILV membranes, although it is difficult to ascertain this because of a lack of contrast. In addition, we have recognized different stages of MVB formation, such as inward budding of the limiting membrane of MVBs and generation of $LBPA^+$ ILVs.

Eosinophils have a mechanism that governs the selective secretion of preformed granules in response to differing stimuli.[3,27,41] We used IFN-γ as an appropriate inductor of eosinophil secretion because it induces specific and regulated secretion of primary vesicles.[39] We selected 10 minutes of stimulation because at this time, the transfected GFP-CD63 appears in the plasma membrane of the eosinophils in our time-lapse analysis. Tetraspanin CD63 is weakly expressed in the Golgi apparatus and undergoes enrichment in granules (MVBs, late endosomes, and others). For this reason, CD63 appears in the plasma membrane as a consequence of MVB fusion after induction of degranulation.[47,48] Our results are fully compatible with those of Mahmudi-Azer et al,[42] although these authors did not provide any data regarding the molecular basis responsible for the observed translocation of CD63 to the plasma membrane. In addition, fusion with the plasma membrane of MVBs/GFP-$CD63^+$ would indicate that probably their ILVs are being secreted as eosinophil exosomes, as we show here.

The viability of eosinophils after IFN-γ treatment was analyzed to determine whether eosinophils release only secretory vesicles and no other granule population that could affect the viability of the cell during stimulation (data not shown).

We have also investigated the capacity of IFN-γ to promote new MVB generation in eosinophils. Our results demonstrated that IFN-γ is not able to induce *de novo* MVBs. There is a time-dependent decrease of LBPA fluorescence intensity by using microscopic analysis and also LBPA/CD63 fluorescence was decreased when measured by using flow cytometric analysis.

Once it was established that eosinophils contain MVBs, we identified and characterized the exosomes that they generate by using different methods. First, we identified eosinophil exosomes using WB; for this purpose, we used CD63, CD9, and Alix (specific exosome proteins included in the list of the top 25 proteins most often identified in exosomes; exocarta.org/exosome_markers). The selection of these proteins was done based on their relation with the endosome pathway and MVB generation. In this study we were able to confirm the presence of exosomes in the eosinophil supernatant and provide the first evidence that eosinophils secrete exosomes. Furthermore, WB has shown an increase in the amount of these proteins after induction with IFN-γ, indicating that this stimuli, a potent secretagogue, triggers eosinophil degranulation. Eotaxin is an excellent stimulus for human eosinophils, which is relevant to asthma or T_H2-type immune responses[26]; nevertheless, eotaxin does not appear to increase $Alix^+$ and $CD63^+$ exosomes, whereas CD9 expression might increase. CD9 is an intracellular and cell surface protein abundant in eosinophils to such an extent as to be considered an eosinophil marker.[49] In fact, CD9 serves as a molecule that delivers stimulation signals on eosinophils.[50] Several authors have found decreasing CD9 expression in eosinophils on activation, a phenomenon probably caused by release/shedding of soluble forms of CD9 and not caused by intracellular degradation.[51,52] It is likely that this CD9 expression decrease could be due to secretion of $CD9^+$ eosinophil exosomes, and for this reason, we have observed an increase in $CD9^+$ exosomes after eotaxin eosinophil stimulation.

Second, we measured the size of the eosinophil exosomes using NanoSight LM10. The size we obtained corresponds to the size range of exosomes acceptable for nanotracking analysis.[53,54] The apparently different size observed by using TEM is due to the fact that, during fixation, shrinkage artifacts for TEM lead to undersizing of the exosomes. Thus in accordance with our results, Ludwig and Giebel[7] observed that vesicles from the same probe appear smaller when prepared for TEM against nanotracking analysis technology.[54,55]

However, the unequivocal characterization of exosomes was performed by using TEM, and therefore we used this method to uncover the exosome presence in the eosinophil supernatant. The structural shape we have seen with TEM had the typical double-membrane characteristic of exosomes and also positive labeling of Alix and CD63. Thus our results show that in eosinophils degranulation of pre-existing MVBs occurs, but we did not detect *de novo* synthesis of MVBs.

Eosinophils are the main effector cells in asthmatic patients. Among the characteristics of asthma of importance are the increased levels of eosinophils and their granule proteins, which are implicated in the exacerbation of asthma pathogenesis.[56] Notably, we have shown that production of eosinophil exosomes is greater in asthmatic patients than in healthy subjects and that these exosomes are functional because they contain molecules relevant to human asthma, such as EPO, MBP, and ECP. Furthermore and in concordance with our data, these eosinophil proteins are included in the Exocarta

database (exocarta.org/exosome_markers). Because MBP, ECP, and EPO expressions are similar in healthy subjects and asthmatic patients, the only real difference is that asthmatic patients have more exosomes than healthy subjects, which presumably could lead to more serious problems when the exosomes are stimulated to release their contents. We hypothesized that the difference in exosome production we found could be attributed to the overstimulation that eosinophils from asthmatic patients have experienced during the development of the illness. This evidence implies that eosinophil exosomes might play an important role in the development of asthma, possibly as a result of the different factors they carry. The difference in eosinophil exosome production between asthmatic patients and healthy subjects gives us a possible parameter to discriminate asthma and asthma phenotypes, although more studies are necessary. The finding of an indicator of illness in eosinophil exosomes could be considered a possible biomarker for the diagnosis of asthma.

Recently, several publications have dealt with the relationship between exosomes and asthma-related diseases.[23,24,57,58] Nevertheless, the origin of the exosome has never been examined in any of these studies. Our data support the idea that it is necessary to know the cellular origin of exosomes because depending on this origin, exosomes have different functions. In addition, the fact that MVB degranulation underlies the observed exosome secretion in eosinophils points out that study of the signals involved in the late stages of MVB traffic could provide clues regarding the observed differences in asthmatic patients. Exosomes could be involved in the inflammatory response because they carry phospholipases and prostaglandins[59] and are implicated in the production of cytokines and leukotrienes in patients with allergic asthma.[24] Until now, the cells that mainly contribute to exosome production in asthma-related diseases were unknown. In this work we have demonstrated that eosinophils contribute to the exosome pool, although others cells can do so as well. It is also necessary to explore the possible mechanisms of asthma in which eosinophil exosomes could be implicated. This knowledge will help us to evaluate them not only as good biomarkers of the disease but also as a new therapeutic tool.

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Key messages

- **Eosinophils are the main effector cells in asthma pathogenesis, and here we describe that eosinophils are able to secrete exosomes.**
- **Eosinophils from asthmatic patients produce higher exosome levels than those from healthy subjects, and therefore exosomes can be considered asthma biomarkers.**

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Video E1. Three of 3 viable GFP-CD63^{+} eosinophils treated with IFN-γ were analyzed and showed that translocation to the plasma membrane began at 8 minutes. The transmittance video shows viable cells.

Video E2. Video capture of an example of purified exosomes by using Nanoparticle Tracking Analysis. Exosome preparations was analyzed by measuring the rate of Brownian motion with the NanoSight LM10 system, which is equipped with fast video capture and particle-tracking software.