Exosomes from Bronchoalveolar Fluid of Tolerized Mice Prevent Allergic Reaction[1]

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Exosomes are nanovesicles originating from multivesicular bodies that are secreted by a variety of cell types. The dual capability of exosomes to promote immunity or to induce tolerance has prompted their clinical use as vehicles for vaccination against different human diseases. In the present study, the effect of allergen-specific exosomes from tolerized mice on the development of allergen-induced allergic response was determined using a mouse model. Mice were tolerized by respiratory exposure to the olive pollen allergen Ole e 1. Exosome-like vesicles were isolated from bronchoalveolar lavage fluid of the animals by the well-established filtration and ultracentrifugation procedure, characterized by electron microscopy, Western blot, and FACS analyses, and assessed in a prophylactic protocol. To this end, BALB/c mice were intranasally treated with tolerogenic exosomes or naive exosomes as control, 1 wk before sensitization/challenge to Ole e 1. Blood, lungs, and spleen were collected and analyzed for immune responses. Intranasal administration of tolerogenic exosomes inhibited the development of IgE response, Th2 cytokine production, and airway inflammation—cardinal features of allergy— and maintained specific long-term protection in vivo. This protective effect was associated with a concomitant increase in the expression of the regulatory cytokine TGF-β. These observations demonstrate that exosomes can induce tolerance and protection against allergic sensitization in mice. Thus, exosome-based vaccines could represent an alternative to conventional therapy for allergic diseases in humans. *The Journal of Immunology,* **2008, 181: 1519–1525.**

Allergy is a major public health problem that affects the quality of life of millions of children and adults, and its prevalence has dramatically increased in many countries, particularly in industrialized ones, where it affects more than 25% of the population (1). In Mediterranean countries (2) and some areas of America, South Africa, Japan, and Australia, one of the most common causes of pollinosis is olive tree (*Olea europaea*) pollen. The main allergen of olive tree pollen is Ole e 1 (3), which has been described as a diagnostic marker for sensitization to *Oleaceae* pollens (4). Therefore, Ole e 1 has been selected as a model allergen for the development of novel therapeutic strategies for allergy.

Currently, allergen-specific immunotherapy is the only curative treatment available for allergic patients that results in immunological allergen unresponsiveness. Even though this treatment can offer protection, it faces several problems, such as long duration, anaphylactic side effects, and limited efficacy (5). Consequently, there is a clear need to develop alternative strategies to allergen-specific immunotherapy aiming to reinstate peripheral tolerance against allergens. Mucosal vaccines, which are based on the mucosal tolerance phenomenon, represent an alternative strategy to conventional immunotherapy (1). Studies in mouse models of allergy have revealed promising results on mucosal tolerance induction with allergens or their derivatives in prophylactic as well as therapeutic approaches (see Ref. 1 for review).

Exosomes are nanovesicles (30–100 nm in diameter) released by a variety of cell types, including dendritic cells (DCs),[3] but they have also been isolated from several body fluids, such as serum, bronchoalveolar fluid (BALF), urine, tumoral effusions, sperm (see Refs. 6 and 7 for review), and breast milk (8). They form by inverse membrane budding into the lumen of late endosomes, which results in the formation of multivesicular bodies (MVBs). Fusion of the MVBs with the plasma membrane leads to the release of exosomes into the medium. Several studies have shown that exosomes display immunomodulatory properties, including both immune stimulation and immune suppression (named tolerogenic exosomes), because they act as Ag-bearing vehicles transferring Ags between cells. For example, DC-derived exosomes stimulate efficient antitumor immunity in animal models (9), and have already been successfully assessed in two phase I clinical trials for the treatment of patients with melanoma or with non-small cell lung carcinoma (10, 11). Regarding their immunosuppressive effects, recent studies have shown that exosomes from modified DCs can suppress both a delayed-type hypersensitivity response and collagen-induced arthritis in mouse models (12, 13). In addition, it also has been demonstrated that exosomes are able to induce donor-specific allograft tolerance (14). Also, tolerosomes, corresponding to exosome-like structures, are produced by intestinal epithelial cells and can induce tolerance to oral Ags (15, 16). On the basis of these and other studies, exosomes have been proposed as vehicles for vaccination against different human diseases. To date, there is just one report showing that B cell-derived exosomes

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[3] Abbreviations used in this paper: DC, dendritic cell; BALF, bronchoalveolar lavage fluid; EM, electron microscopy; Exo_{Con}, exosomes isolated from naive mice; Exo_{Tol}, exosomes isolated from mice tolerized against Ole e 1; i.n., intranasal; MVB, multivesicular body; SP-B, surfactant protein B.

can present allergen-derived peptides and stimulate allergen-specific T cells, suggesting that exosomes could play a role in allergy (17). Following the concept of tolerogenic exosomes, it would be interesting to investigate whether such vesicles could be the basis for a novel form of mucosal allergy vaccine for prophylaxis and therapy of individuals at risk or patients.

In the present study, the effect of allergen-specific exosomes from tolerized mice on the development of allergen-induced allergic response was determined using a mouse model. We demonstrate that intranasal (i.n.) administration of tolerogenic exosomes inhibited the classical pathology associated with allergy, namely IgE response, Th2 cytokine production, and airway inflammation.

Materials and Methods

Abs and reagents

Abs used for exosome characterization by Western blotting are as follows: rabbit anti-mouse MFGE-8 (lactadherin) serum prepared as previously described (18); monoclonal anti-mouse Alix/AIP1 (49 clone) and clathrin (23 clone) (BD Transduction Laboratories); mouse anti-flotillin-1 (18 clone) and rat monoclonal anti-mouse CD9 (KMC8 clone; BD Pharmingen); mouse anti-ICAM-1 (YN1/1.7.4 clone; Beckman Coulter); goat anti-tsg 101 (M-19 clone; Santa Cruz Biotechnology); hsc70 (1B5 clone) and gp96 (9G10 clone) (StressGen Biotechnologies); rabbit anti-sheep surfactant protein B (SP-B) polyclonal Ab (Chemicon International); and rabbit anti-Ole e 1 allergen serum prepared as previously described (19). HRP-conjugated secondary Abs were from Pierce. Abs used for FACS analysis were obtained from BD Pharmingen, as follows: FITC-conjugated anti-mouse I-A^d, H-$2K^d$; biotin-conjugated anti-mouse CD9 and ICAM-1; and PerCP-conjugated streptavidin.

Ole e 1 was purified from olive pollen, as described (3).

Mice

Female BALB/c mice (6–8 wk old) were obtained from Harlan Interfauna Ibérica. Animals were maintained at the Animal Care Services of the Faculty of Biology (Complutense University), according to the local guidelines for animal care and use. Experiments were approved by the Animal Experimentation Ethics Committee of Complutense University.

Sensitization and airway challenge

Mice were immunized by i.p. injection with 1 μg of Ole e 1 in 2 mg of alum, in a volume of 150 μl of PBS, twice at week intervals. After 7 days, mice received i.n. 10 μg of Ole e 1 in 20 μl of PBS on 3 consecutive days under anesthesia (19).

Prophylactic protocol

In a pilot experiment, several i.n. pretreatments were performed to find the optimal dose and time of Ag administration. Immunomodulation was achieved by i.n. application of 5 μg of exosomal proteins divided on 3 doses on 3 consecutive days, and, therefore, it was chosen as the optimal protocol in the present study. Anesthetized mice were i.n. pretreated with exosomes from tolerized (Exo_{Tol}) or naive mice (Exo_{Con}). One week later, mice were sensitized, as described above (short-term protocol). For long-term experiments, Ole e 1 sensitization was started 10 wk after treatment. The experiments were performed with seven to eight mice/group and repeated two or three times to ensure reproducibility.

Induction of i.n. tolerance, collection of BALF, and exosome purification

Mice lightly anesthetized with ketamine/xylazine received i.n. Ole e 1 (100 μg) in 20 μl of PBS on 3 consecutive days (days 0, 1, and 2). Control mice received i.n. PBS. BALF was taken 24 h later (day 3). Animals were injected i.p. with a lethal dose of pentobarbital. After trachea canulation, the lungs were lavaged with 1 ml of PBS three times, and the fluids were pooled.

Exosomes were isolated from BALF of tolerized or naive mice, as described previously, for exosomes (20). The protein content in exosome preparations was measured by the microbicinchoninic acid assay (Pierce Chemical). Exosomal proteins (10, 15, or 30 μg) were analyzed by SDS-PAGE and Western blotting or coated on beads for FACS analysis, as described (20).

Electron microscopy (EM)

Exosomes fixed in 2% paraformaldehyde were loaded on copper-formwar/carbon-coated EM grids. Samples were contrasted in 2% uranyl acetate and observed at 80 kV with a JEOL1010 transmission electron microscope.

Sucrose gradient

Flotation of exosomes was performed on a linear 2 M to 0.25 M sucrose gradient by 18-h centrifugation at 100,000 $\times$ *g*, as described (20). Pellets recovered from 0.5-ml fractions were analyzed by EM.

Lung histology

Lungs (n = 2–3 mice/group) were removed, washed with PBS, fixed in 10% formalin, and embedded in paraffin. Sections (5 μm) were cut and stained with H&E for light microscopy. For each mouse, 10 airway sections, randomly distributed through the left lung, were assessed for severity/frequency of inflammatory response by morphometric analysis, by individuals blinded to the protocol design (semiquantitative scale ranging from 0 to 5^+).

ELISA

Blood samples were collected from the retro-orbital plexus 7 days after the last immunization (terminal bleeding). Individual sera were prepared and stored at −20°C until used. Ole e 1-specific Abs were measured by ELISA, as previously described (21). Serum samples were diluted 1/25,000 for IgG1 and 1/400 for IgG2a. Ab levels were expressed as OD values at 492 nm. Total IgE (diluted 1/80) Abs were measured by a sandwich ELISA using the OptEIA mouse IgE set (BD Pharmingen), according to the manufacturer's instructions. In our mouse model, it has been shown that specific IgE levels in serum can be estimated by measuring total IgE (21).

Proliferation and cytokine assays

Seven days after the last immunization, spleens from Ole e 1-sensitized mice were removed aseptically, minced, and filtered through sterile filters, and a cell suspension was prepared, as described (21).

For T cell proliferation assays, spleen cells (2×10^5 cells/well, 0.2 ml/well) were stimulated with Ole e 1 (2 μg/ml) for 96 h in 96 round-bottom plates (Costar). The cultures were pulsed with 1 μCi/well [^{3}H]thymidine (Amersham) during the last 16 h of culture. Cells were harvested on microbeta filter mats using a 96-well harvester, and proliferative responses were measured by beta plate scintillation counting. The stimulation index was calculated as the ratio of cpm of the unstimulated control and allergen-stimulated cells.

To measure cytokine production, spleen cells were cultured in 48-well plates (Costar) at 10^7 cells/ml (0.5 ml/well) and stimulated with Ole e 1 (2 μg/ml). Supernatants were taken after 48 or 72 h and stored at −20°C until analysis. Levels of IL-4, IL-5, IL-10, and IFN-γ were measured by ELISA kits (BD Pharmingen). Values in pg/ml were calculated according to standard curves obtained with recombinant mouse cytokines. The sensitivity of the assays was 5 pg/ml for IL-4, 60 pg/ml for IL-5, 15 pg/ml for IL-10, and 25 pg/ml for IFN-γ.

RNA isolation and real-time PCR assays

Lungs and spleens were homogenized, and total RNA was isolated using RNAqueous-4PCR kit (Ambion Europe), according to the manufacturer's instructions. Reverse transcription was done using Superscript first-strand synthesis system for real-time PCR (Invitrogen). Primers specific for IL-10 (forward, 5′-TGCCAAGCCTTATCGGAAAT-3′; reverse, 5′-TTTCTGGGC CATGCTTCTCT-3′), TGF-β (forward, 5′-CACCGGAGAGCCCTGGATA-3′; reverse, 5′-GCCGCACACAGCAGTTCTT-3′), and β-actin (forward, 5′-CACAGCTGAGAGGGAAATCGT-3′; reverse, 5′-GCCATCTCCTGCTCG AAGTCTA-3′) were designed and synthesized by Sigma-Aldrich. Real-time analysis was performed on an ABI Prism 7900 (Applied Biosystems) using SYBR Green PCR Master Mix for real-time PCR (Applied Biosystems). After normalization of the data according to the expression of β-actin mRNA, relative levels of cytokine mRNA were calculated using the $\Delta\Delta C_T$ method.

Statistical analysis

Data are given as means ± SEM from two (long-term) or three (short-term) independent experiments. Statistical analyses were performed by using Mann-Whitney U test (nonparametric) or Student's t test (parametric).

Results

Characterization of mouse BALF-derived exosomes

To examine the immunosuppressive properties of exosomes, a mouse model of tolerance to the allergen Ole e 1 was established,

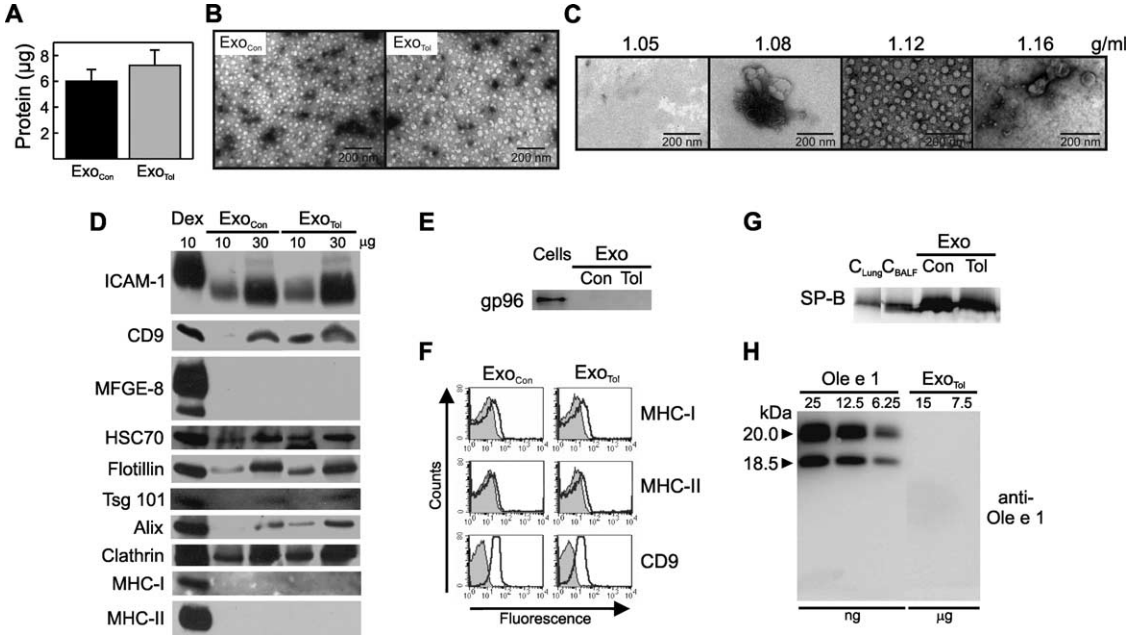

FIGURE 1. BALF-derived exosomes from tolerized mice are similar to those derived from naive mice. *A*, Relative exosome production in tolerized (Exo_{Tol}) vs naive mice (Exo_{Con}) calculated as μg of total exosomal protein per mouse. Data are expressed as mean ± SEM ($n = 20$ mice/group) from eight independent experiments. *B*, Electron micrographs of exosomes from tolerized or naive mice. *C*, Analysis of exosomes on sucrose gradient by EM. *D*, Western blot analysis for the presence of exosomal proteins in BALF-derived exosomes compared with those derived from DC (Dex). *E*, Similar experiment for the expression of gp96 in BALF cell lysate (30 μg) and both types of exosomes (30 μg). *F*, Flow cytometric analysis for the surface expression of MHC-I, MHC-II, and CD9 in exosomes from BALF. Shaded histograms indicate isotype controls. *G*, Western blot analysis for the presence of SP-B in BALF-derived exosomes (15 μg) compared with lung (C_{Lung}) and BALF (C_{BALF}) cell lysates (15 μg). *H*, Presence of Ole e 1 in Exo_{Tol}. Bands correspond to the glycosylated (20-kDa) and nonglycosylated (18.5-kDa) forms of Ole e 1.

involving repeated i.n. administration of Ole e 1 (our unpublished data). Exosome-like vesicles were isolated from BALF of tolerized (Exo_{Tol}) or naive animals (Exo_{Con}), as control, by the well-established filtration and ultracentrifugation procedure (20). To confirm that the vesicles recovered were typical exosomes, they were characterized by EM, flotation on a sucrose gradient, Western blot, and FACS analysis. Typically, 5–8 μg of exosomal proteins was isolated per mouse. No significant differences were detected in the purification yield of BALF-derived exosome-like vesicles from tolerized mice compared with naive animals (Fig. 1*A*). EM analysis of BALF-derived Exo_{Tol} showed round-shaped vesicles of 30–45 nm in diameter (Fig. 1*B*). These vesicles were morphologically similar to those isolated from naive mice. Exosome-like vesicles floated on a continuous sucrose gradient at density close to 1.12 g/ml (Fig. 1*C*). No exosome-like vesicles were detected on lower or higher density fractions. Western blot (Fig. 1, *D* and *E*) and FACS (Fig. 1*F*) analyses showed that BALF-derived exosome-like vesicles from mice tolerized to Ole e 1 and control mice were positive for typical exosomal proteins, especially those involved in MVB formation such as tsg101 and Alix, and also contained proteins identified in the well-characterized DC-derived exosomes (22), i.e., MHC-I (Fig. 1*F*), ICAM-1, CD9, hsc70, flotillin, and clathrin (Fig. 1*D*), but negative for proteins not found in exosomes, such as gp96, a resident protein of endoplasmatic reticulum (Fig. 1*E*). We also observed that BALF-derived exosome-like vesicles expressed no detectable levels of MHC-II (Fig. 1*F*), nor MFGE-8 (Fig. 1*D*), two abundant proteins on DC-derived exosomes, suggesting that DCs are not the major source of the vesicles present in BALF: other airway cells could be the sources of BALF-derived exosomes.

To analyze whether epithelial lung cells were the source of BALF-derived exosomes, the expression of SP-B, a specific surfactant protein that is synthesized in alveolar type II cells (23), on BALF-derived exosomes was analyzed by Western blot (Fig. 1*G*). SP-B was identified in lung and BALF cells, as expected, and on both types of BALF-derived exosomes. This result shows that type II cells are the main source of BALF-derived exosomes.

The presence of Ole e 1 in Exo_{Tol} was analyzed by Western blot using a polyclonal Ab against this allergen (Fig. 1*H*). No detectable

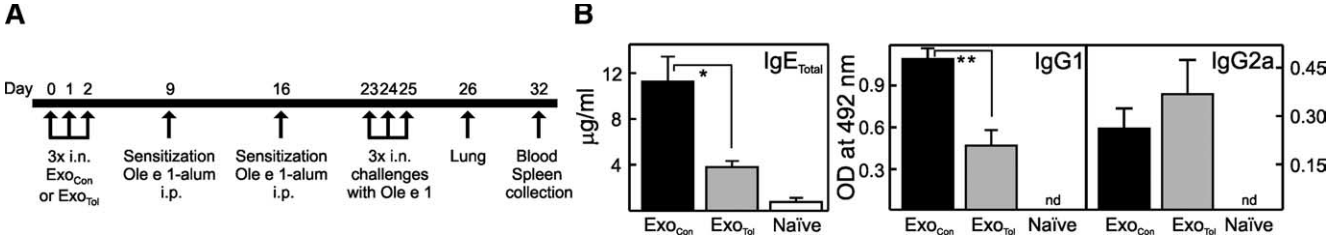

FIGURE 2. BALF-derived Exo_{Tol} inhibit synthesis of IgE and induce IgG2a in a mouse model of allergic sensitization. *A*, Experimental protocol used. *B*, Serum levels of Ole e 1-specific IgE, IgG1, and IgG2a determined by ELISA. Data are expressed as means ± SEM ($n = 15$ mice/group) from three separate experiments. *, $p < 0.001$ and **, $p < 0.001$ compared with sham-pretreated mice. Exo_{Con}, animals pretreated with naive exosomes; Exo_{Tol}, mice pretreated with tolerogenic exosomes.

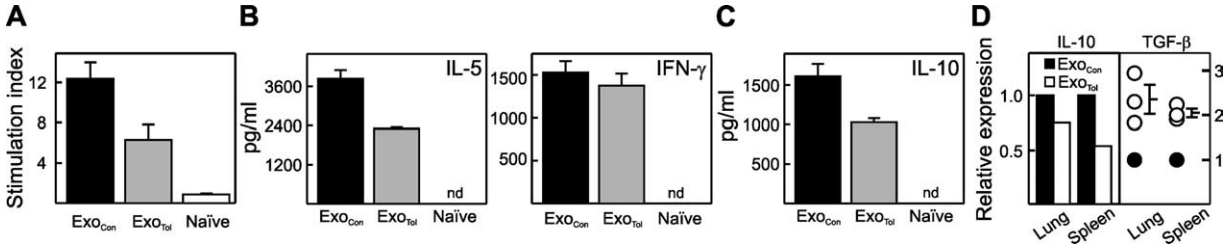

FIGURE 3. T cell reactivity to Ole e 1 in spleen cell cultures from mice pretreated with Exo_{Tol}. *A*, Proliferate responses. Data are expressed as means ± SEM ($n = 5$ mice/group). IL-5 and IFN-γ (*B*) and IL-10 (*C*) production determined by ELISA. Results are shown as means ± SEM ($n = 5$ mice/group). *D*, Expression of IL-10 and TGF-β mRNA in lung and spleen as determined by RT-PCR. Values were normalized over those of the housekeeping gene actin, and expressed as a fold change, compared with Exo_{Con}-pretreated mice defined as 1. For TGF-β mRNA, data of three independent experiments are presented as scattered plot. Data are representative of three independent experiments.

levels of the allergen, as intact protein or as large processed fragments, were found in Exo_{Tol}.

Therefore, although the size and density of BALF-derived vesicles are slightly smaller (30–45 nm instead of 50–100 nm) and lower (1.12 g/ml instead of 1.13–1.20 g/ml), respectively, than those generally described for exosomes (20), these values have been reported for other exosomes (24). Furthermore, our vesicles display the typical protein composition of exosomes, especially a signature enrichment in MVB-associated proteins. Thus, we will refer to these vesicles as BALF-derived exosomes throughout this work.

Tolerogenic exosomes inhibit both specific IgE and IgG1 Abs in an allergic sensitization model

We examined whether BALF-derived exosomes from Ole e 1-tolerized mice may serve as a preventive vaccine for allergy in a mouse model of allergy induced by Ole e 1/alum sensitization, followed by airway challenges. Mice were i.n. pretreated with tolerogenic or naive exosomes, as sham-pretreated controls, before sensitization with Ole e 1, and the allergen-specific response was analyzed (Fig. 2*A*). Sham-pretreated animals developed an allergic state comparable to that observed in untreated mice sensitized and challenged with the allergen (data not shown). Intranasal pretreatment with Exo_{Tol} reduced IgE Ab levels as compared with the sham-pretreated controls (Fig. 2*B*). A reduction of specific IgG1 Ab levels was also observed in Exo_{Tol}-pretreated animals. By contrast, specific IgG2a Ab levels were not significantly changed by the pretreatment with Exo_{Tol} compared with the sham-pretreated group.

Pretreatment with tolerogenic exosomes inhibits Th2 cytokines, but induces TGF-β

To determine the effect of the pretreatment with Exo_{Tol} in the T cell response, cytokine production (IL-4, IL-5, and IFN-γ) and lymphoproliferative response were measured in pooled spleen cell cultures with or without Ole e 1.

The lymphoproliferative response to Ole e 1 was inhibited in the cultures of Exo_{Tol}-pretreated mice compared with that of sham-pretreated group (Fig. 3*A*). IL-5 production was significantly reduced in spleen cell cultures of mice pretreated with Exo_{Tol} in comparison with the sham-pretreated controls (Fig. 3*B*). No significant changes were detected in IFN-γ levels in spleen cell cultures of these animals. In these experiments, IL-4 could not be detected in vitro under similar conditions, probably due to either insufficient secretion or too rapid use by these cells.

To determine whether suppression of experimental allergy in Exo_{Tol}-pretreated mice was associated with the production of regulatory cytokines, the levels of IL-10 in spleen cell cultures were determined by ELISA. As shown in Fig. 3*C*, IL-10 production was decreased in mice pretreated with Exo_{Tol} as compared with the control group. In addition, TGF-β and IL-10 mRNA expressions were determined by RT-PCR in lung and spleen tissues. Mice i.n. pretreated with Exo_{Tol} displayed a 2-fold increase of TGF-β mRNA in lung and spleen tissues compared with sham-pretreated controls (Fig. 3*D*). Regarding IL-10, mRNA levels were reduced compared with those of sham-pretreated controls in both tissues.

Tolerogenic exosomes reduce allergen-induced airway inflammation in mice

To assess the effects of the i.n. pretreatment with Exo_{Tol} in airway inflammation, lung histology was examined for each experimental group 24 h following the last i.n. Ole e 1 challenge. Histological examination revealed that i.n. pretreatment with Exo_{Tol} significantly reduced inflammatory cell infiltration into the lung after Ole e 1 challenge compared with sham-pretreated controls (Fig. 4).

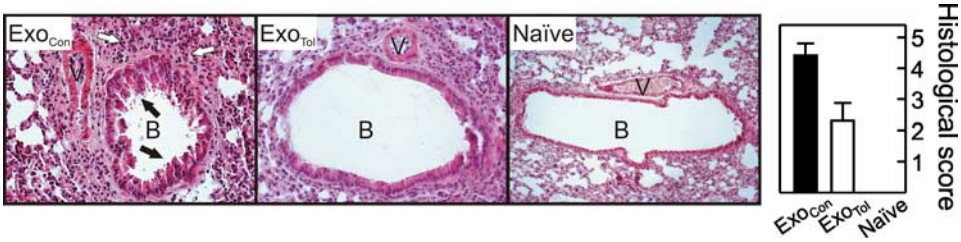

FIGURE 4. Exo_{Tol} pretreatment inhibits airway inflammation. Representative lung sections stained with H&E. Exo_{Con}, lung parenchyma from Ole e 1-sensitized mouse that has received Exo_{Con}, showing numerous inflammatory cells surrounding the airways and vessels (open arrow), and bronchiolar epithelium showing hyperplastic columnar epithelial cells (filled arrows); Exo_{Tol}, bronchiolar mucosa consist of low cuboidal epithelium with minimal cellular infiltration and scarce eosinophils of an Ole e 1-sensitized mouse that has received Exo_{Tol}; Naive, lung tissue from untreated control mouse showing normal airway and surrounding parenchyma, and absence of peribronchiolar inflammatory infiltrates. Magnifications: ×20 (Exo_{Tol} and Exo_{Con}); ×10 (Naive). Data are representative of three independent experiments ($n = 2$–3 mice/group). B, bronchium; V, blood vessel.

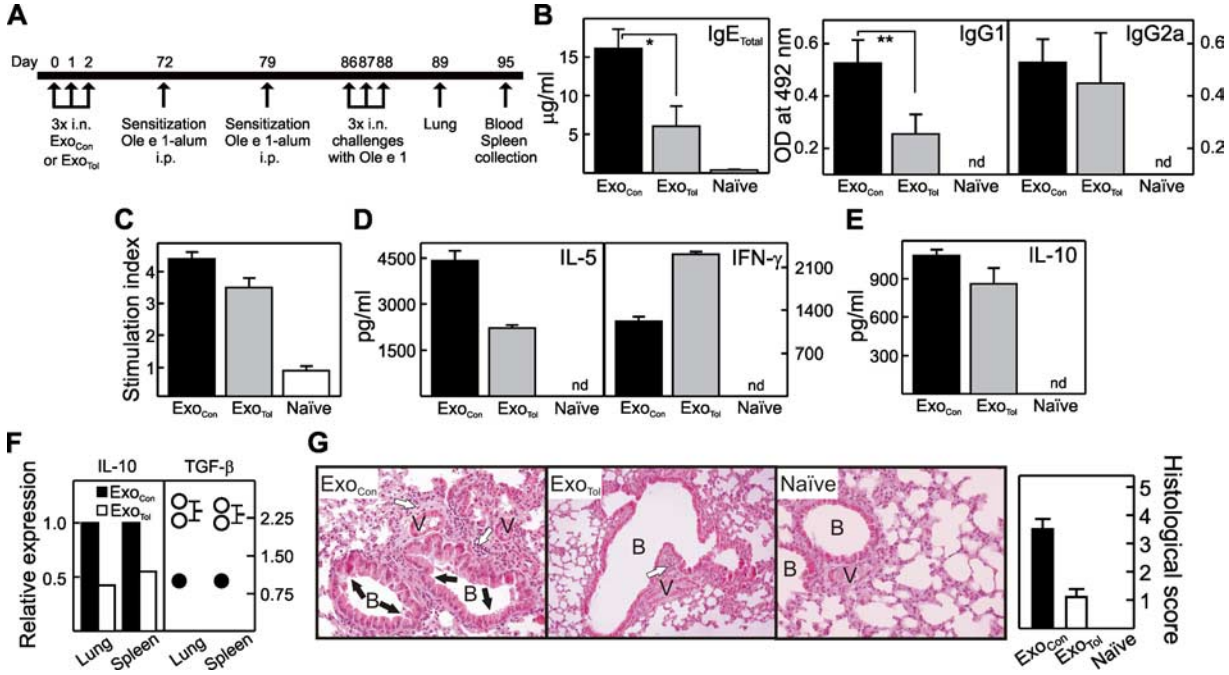

FIGURE 5. Exo$_{Tol}$ pretreatment induces long-lasting protection against Ole e 1 sensitization. *A*, Experimental protocol used. *B*, Serum levels of Ole e 1-specific IgE, IgG1, and IgG2a determined by ELISA. Data are expressed as means ± SEM ($n = 5$ mice/group). *, $p < 0.03$ and **, $p < 0.05$ compared with sham-pretreated mice. Proliferative response (*C*) and cytokine production (*D* and *E*) in spleen cell cultures stimulated in vitro with Ole e 1. Results are shown as means ± SEM ($n = 5$ mice/group). *F*, Expression of IL-10 and TGF mRNA in lung and spleen after i.n. pretreatment with Exo$_{Tol}$ determined by RT-PCR. Values were normalized over those of the housekeeping gene actin, and expressed as a fold change, with values from Exo$_{Con}$-pretreated mice defined as 1. For TGF-β mRNA, data of two independent experiments are presented as scattered plot. *G*, Representative photomicrographs of lung sections from each group ($n = 2$ mice/group). Magnifications: ×20 (Exo$_{Tol}$ and Naive); ×10 (Exo$_{Con}$). Open arrows, areas with cellular infiltration. Filled arrows, hyperplastic bronchiolar epithelium. B, bronchium; V, blood vessel. Data are representative of two independent experiments.

The influx of eosinophils into the lung was suppressed for 50% by exosome pretreatment. No mucus secretion was observed.

Tolerogenic exosomes induce long-lasting protection against allergic sensitization

To examine whether exosome pretreatment induces a suppressive memory response, animals were sensitized 10 wk after pretreatment (Fig. 5*A*).

Ole e 1-specific Ab levels in serum. In the long-term protocol, Exo$_{Tol}$ pretreatment suppressed serum-specific IgE and IgG1 Ab levels to the same extent as after short-term protocol compared with sham-pretreated controls (Fig. 5*B*). No significant changes were observed in specific IgG2a Ab levels after a long-term interval.

Proliferative response and cytokine production. In mice that received Exo$_{Tol}$ pretreatment, proliferative response was suppressed compared with that of the sham-pretreated controls (Fig. 5*C*). Intranasal pretreatment with Exo$_{Tol}$ resulted in a significant reduction of IL-5 levels (Fig. 5*D*), whereas a decrease was detected on IL-10 production (Fig. 5*E*). In contrast, IFN-γ was increased in cell cultures of mice i.n. pretreated with Exo$_{Tol}$ compared with the sham-pretreated mice (Fig. 5*D*).

IL-10 and TGF-β mRNA. As Fig. 5*F* shows, after long-term protocol, Exo$_{Tol}$ pretreatment also increased TGF-β mRNA expression, compared with sham-pretreated controls, in both lung and spleen. IL-10 mRNA levels were reduced compared with those of i.n. sham-pretreated controls.

Airway inflammation. After a long-term protocol as well as after short-term, lung cell infiltration was decreased as compared with sham-pretreated mice, and this was accompanied by an inhibition of eosinophil influx (Fig. 5*G*).

From these data, it can be inferred that Exo$_{Tol}$ pretreatment induced a suppressive memory response, and TGF-β may be a mediator of this effect in this mouse model of allergy.

Discussion

In this study, we have demonstrated that i.n. administration of BALF-derived Exo$_{Tol}$ induces tolerance and protects against allergic sensitization. One of the main advantages of the i.n. exosome-based vaccine is its potentiality to suppress two important clinical features of allergy, as follows: IgE Ab levels in serum and airway inflammation.

BALF cells appeared to secrete exosome-like vesicles as defined by morphological and biochemical criteria. Exosome-like vesicles were homogenous in size and floated with a sucrose gradient of 1.12 g/ml, which fits with the density reported for exosomes (1.11–1.21 g/ml) (24). Moreover, BALF-derived exosomes bear MHC-I, tetraspanin CD9, hsc70, flotillin, tsg101, Alix, and clathrin, a defined set of cellular proteins found in exosomes produced by other cells, but no MFGE-8 (for review, see Ref. 25). These data allowed us to identify these BALF-derived vesicles as exosomes. BALF-derived exosomes from mice show differences in protein content compared with DC-derived exosomes. BALF-derived exosomes showed low levels of MHC-I and do not appear to express MHC-II, at least with the detection limits of Western blot and flow cytometry used in this study. It was unexpected that BALF-derived exosomes from mice do not bear MHC class II molecules because these molecules have been found in exosomes from human BALF (26). This is supported by the presence of MHC class II$^{low/-}$ exosomes reported in human breast milk (8). They also showed SP-B, a specific protein of pulmonary surfactant that is synthesized in alveolar epithelia type II cells (23), suggesting that these cells could be the main source of BALF-derived exosomes. In addition

to its function in the regulation of lung homeostasis, type II cells have broad-ranging roles in the modulation of the activity of adjacent cell populations, including immune cells (27). Most recently, Lo et al. (28) have proposed that alveolar epithelia type II cells protect the lung from unwanted inflammatory responses, such as allergic reactions, by tolerizing T cells to potentially innocuous Ags as allergen. Based on these studies and taking into account the immunomodulatory properties of exosomes and their presence in human BALF, we proposed that type II cells, indirectly, can regulate the immune response in the lung by releasing exosomes. Exosomes released from different cellular sources may also have a role in allergy (for review, see Ref. 29). B cell-derived exosomes can present allergen-derived peptides and stimulate allergen-specific T cells, suggesting that exosomes could play a role in allergy (17). Furthermore, it has been suggested that exosomes from mast cells may regulate the allergic immune response by the restoration of the Th1/Th2 balance (for review, see Ref. 30), because they preferentially induce Th1-type responses (30–32). In this context, it deserves to mention that immunosuppressive effects mediated by mast cells have been reported in a delayed-type hypersensitivity response model (33).

The prophylactic capacity of exosomes produced by BALF cells was tested in the mouse model of Ole e 1-allergic sensitization. Mice pretreated with tolerogenic exosomes were protected against subsequent sensitization/challenge with the allergen and maintained long-term specific protection. The observed prophylactic effect is not due to contamination in the exosome preparation because control exosomes derived from naive mice were not able to confer any prophylactic effect. The efficiency of exosomes in preventing Ole e 1 sensitization could be due to both the presence of allergen and/or allergen-derived peptides on exosomes (Ag specific) and the exosome phenotype (nonspecific). Thus, if Ole e 1 is present in exosomes, it must be at level below detection threshold, and/or as processed form, such as MHC-I- or hsc70-peptide complexes. In this respect, it has been proposed that heat shock protein-peptide complexes play a role in cross-presentation (for review, see Ref. 34). Exosomes may be involved in both direct and cross-presentation of Ags to T cells. Because exosomes act as a system of antigenic spreading and amplification of immune response, this could explain why low amounts of antigenic peptides on exosomes are highly efficient in prevention against allergic sensitization. The fact that Exo_{Tol}, but not Exo_{Con}, protects mice from allergic sensitization allows us to suggest that the tolerogenic effect of Exo_{Tol} is due to intrinsic properties of these exosomes, rather than to the i.n. route of administration. Preliminary studies performed by our group using the same prophylactic approach support this idea; we found that i.n. pretreatment with BALF-derived exosomes from sensitized mice exacerbated specific Th2 response instead of suppressing it as Exo_{Tol} does (N. Prado, C. Théry, M. Villalba, R. Rodríguez, and E. Batanero, unpublished data). These results are in agreement with the observation that the protein content of exosomes varies depending on the origin and the physiological state of the cell, and these differences can be related to their different functions (22). In addition, the lipid composition of exosomes may also contribute to their immunomodulatory properties because it has been shown that exosomes can also be distinguished by their lipid content (35) and lipids act as adjuvants. Moreover, it would be of interest to investigate whether tolerance induction with Exo_{Tol} is associated with bystander suppression to an unrelated allergen (36).

In this setting, the increased levels of the suppressive cytokine TGF-β are associated with the Exo_{Tol}-mediated suppression effect, suggesting that this immunoregulatory cytokine could play a role in the suppression of the allergic response observed in this model. In addition, IFN-γ could also play a role in the suppression of Th2 immune response and airway inflammation because its levels are high in the tolerized mice. In support of this idea, the immunosuppressive properties of this cytokine have been shown in vivo because it confers protection from autoimmune and allergic diseases (37–40). Although the interplay between IFN-γ and TGF-β, which mediate the suppression of the allergic response in mice that have been pretreated with Exo_{Tol}, remains open, Myers et al. (41) reported that $CD8^+$ regulatory T cells require IFN-γ to generate a TGF-β-based suppression. This hypothesis is the aim of another investigation.

The potent in vivo immunosuppressive effects of tolerogenic exosomes from BALF support the role of these vesicles as Ag-transferring units between immune cells, contributing to amplify specific immune responses. It is possible that BALF-derived exosomes could modulate, either directly or indirectly, the activity of both DCs and T cells, rendering them able to confer protection against allergic sensitization. It is unclear whether exosomes can directly activate T cells, because several studies have indicated that efficient activation of T cells by exosomes requires recapture by APCs (22, 42- 44). The present model should provide a useful tool for addressing these important issues in future research.

The strong immunosuppressive effects on the allergic response point to considering i.n. exosomes-based vaccination as an alternative to treat allergic diseases. In this respect, we have previously described in the mouse model of Ole e 1 sensitization (using the same prophylactic protocol) that i.n. administration of free Ole e 1 or a peptide-T of the allergen, free or encapsulated into poly(lactide-*co*-glycolide) microparticles, prevents allergic sensitization (19, 45). Although all of the prophylactic approaches suppress the most important clinical features of allergy (but using different mechanisms), exosomes have several advantages over the previous reported vaccines. They are acellular structures, but contain a wide array of cellular proteins, some of which modulate immune responses. Exosomes are natural Ag-transferring units between immune cells, allowing cross-presentation and contributing to amplify immune responses. These vesicles are rather stable and reduce the dose of Ag required to induce an immune response. Moreover, exosome display technology permits manipulation of their protein composition and tailoring for different functions (46). Despite the clinical relevance to test the therapeutic effects of Exo_{Tol}, the possibility of using prophylactic allergy vaccines for early prevention in atopic individuals or children at risk has been proposed (1, 47, 48). Further studies are required if prophylactic and therapeutic exosomes-based vaccination strategies are to be developed against allergy.

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Disclosures

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