Active Uptake of Dendritic Cell-Derived Exovesicles by Epithelial Cells Induces the Release of Inflammatory Mediators through a TNF- α -Mediated Pathway

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Dendritic cells (DCs) can release hundreds of membrane vesicles, called exovesicles, which are able to activate resting DCs and distribute antigen. Here, we examined the role of mature DC-derived exovesicles in innate and adaptive immunity, in particular their capacity to activate epithelial cells. Our analysis of exovesicle contents showed that exovesicles contain major histocompatibility complex-II, CD40, and CD83 molecules in addition to tumor necrosis factor (TNF) receptors, TNFRI and TNFRII, and are important carriers of TNF- α . These exovesicles are rapidly internalized by epithelial cells, inducing the release of cytokines and chemokines, but do not transfer an alloantigen-presenting capacity to epithelial cells. Part of this activation appears to involve the TNF- α -mediated pathway, highlighting the key role of DC-derived exovesicles, not only in adaptive immunity, but also in innate immunity by triggering innate immune responses and activating neighboring epithelial cells to release cytokines and chemokines, thereby amplifying the magnitude of the innate immune response. (Am J Pathol 2009, 175:696–705; DOI: 10.2353/ajpath.2009.080716)

Dendritic cells (DCs) are antigen-presenting cells with a unique ability to induce primary immune responses. They are present in trace amounts in most tissues, but they are particularly abundant and act as sentinels in organs with an environmental interface, such as the skin, the respiratory system, and the gastrointestinal tract. Due to their location, immature dendritic cells are profoundly influ-

enced by the environment and transmit danger signals to cells of the adaptive immune system. The presence of pathogens activates immature dendritic cells and triggers their maturation, resulting in enhanced expression of costimulatory molecules such as CD86 and CD80, and of maturation markers such as CD83. Once activated, DCs migrate to lymph nodes where antigen presentation leads to the maturation and proliferation of specific T-cell clones, which in turn migrate to the injured tissue.¹

Depending on their location, DCs are able to release a specific array of cytokines to amplify the innate response. In addition, we would like to suggest that the innate and adaptive immune response may also be amplified through the release of tiny DC-derived microparticles. At least two types of vesicles released from DCs into the extracellular medium have been described. The first type are membrane vesicles, or exovesicles, which are between 0.1 and 1 μ m in diameter; they are produced by membrane surface shedding, and released through a process similar to viral budding.^{2–4} The second type of vesicle is defined as an exosome, ie, microvesicle of endocytic origin, cup-shaped, and $\sim$ 0.05 μ m in diameter; exosomes are released through exocytosis of multivesicular bodies.^{4,5}

Initially, the secretion of these tiny microparticles was described as a process designed to regulate membrane functions and eliminate unnecessary membrane proteins.⁵ However, exosomes have raised immunological interest because they originate from compartments of the endocytic pathway, which are sites of peptide loading on major histocompatibility complex (MHC) class II molecules. Indeed, both exovesicles and exosomes have

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been shown to be highly immunogenic, expressing on their surface not only MHC II molecules, but also costimulatory molecules such as CD86,⁵⁻⁷ and specific proteins lacking secretory signals sequence, such as interleukin (IL)-1 β .⁸⁻¹⁰ Recently, we were able to quantify, on a per cell basis, the release of exovesicles from activated DCs; these exovesicles represent the most relevant microparticles released by DCs. Using double vital staining, we demonstrated that exovesicles released from activated DCs can fuse with the membrane of resting DCs, thereby allowing them to present alloantigens to T-lymphocytes.²

In the present study, we analyzed the composition and the fate of exovesicles regarding the epithelium. We were able to show that exovesicles from lipopolysaccharide (LPS)-activated DCs are important carriers of tumor necrosis factor (TNF)- α . Using double vital staining, we demonstrated that they are internalized by epithelial cells (ECs), and that this process induces the release of inflammatory mediators such as IL-8, Monocyte chemoattractant protein-1 (MCP-1), Macrophage inflammatory protein 1 β (MIP-1 β), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), and TNF- α . Furthermore, we demonstrate that the TNF- α cascade is one of the pathways involved in the activation of these cytokines. In contrast to the well-characterized transfer of alloantigens of exovesicles to heterologous resting DCs, exovesicles in the co-culture with ECs do not transfer an antigen presenting capacity to ECs. Our results demonstrate the potential role of exovesicles not only in adaptive immunity, as a relevant source of antigens fusing with the cytoplasmic membrane of resting DCs, allowing them to present antigens, but also in innate immunity by triggering ECs to release cytokines-chemokines, thereby amplifying the magnitude of the innate immune response.

Materials and Methods

Monocyte Isolation and Differentiation to Dendritic Cells

Monocytes generated from peripheral blood mononuclear cells of healthy human donors were isolated by Ficoll-Hypaque density gradient centrifugation of buffy coats as described previously,¹¹ following spontaneous aggregation¹² and rosetting.¹³ Briefly, Ficoll-Paque-purified peripheral blood mononuclear cells were suspended in RPMI 1640 medium (Invitrogen Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum (Biochrome AG, Berlin, Germany), 2 mmol/L glutamine, 100 U of penicillin per ml, and 100 U streptomycin per ml, referred to as complete culture medium, containing 2 μ g polymyxin B sulfate/ml (Sigma-Aldrich, Buchs, Switzerland). Cells were incubated for 40 minutes at 4°C for aggregation. Rosetting was applied to deplete contaminant lymphocytes. Monocyte enriched fractions were incubated overnight with sheep red blood cells (BioMérieux, Geneva, Switzerland). Monocyte fractions characterized by high expression of CD14 (more than 85%) and low expression of CD83 and CD86 (less than 5%) were then isolated by Ficoll-Hypaque density gradi-

ent centrifugation. Differentiation of DCs from monocytes was performed as originally described by Sallusto and Lanzavecchia,¹⁴ by culture cells in the presence of granulocyte-macrophage colony-stimulating factor (10 ng/ml) and interleukin-4 (10 ng/ml) for 6 days. The cells were kept at 37°C in a 5% CO₂ humidified atmosphere. On day 3, the culture medium was replaced with fresh medium.

Labeling and Stimulation of DCs

After 6 days in culture, DCs were washed and suspended at a density of 1×10^6 cells/ml in serum-free medium (RPMI 1640 medium). Cells were labeled with VIBRANT cell labeling DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C and 5% CO₂. After labeling, cells were washed three times with RPMI 1640 in 37°C pre-warmed media, and cultured in RPMI 1640 supplemented with 1% glutamine and 1% microvesicle-free human serum obtained by ultracentrifugation ($110,000 \times g$) of the serum for 2 hours. Cells were stimulated with 100 ng LPS or left unstimulated and incubated for 12 hours at 37°C in 5% CO₂.

Labeling and Stimulation of ECs

Human A549 (ATCC#CCL185) alveolar epithelial cells were grown in complete culture medium. Eighty-percent confluent cells were harvested by trypsinization, washed twice and resuspended in pre-warmed RPMI 1640 serum-free medium at a density of 1×10^6 cells/ml. Cells were labeled with VIBRANT cell labeling DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) (Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C and 5% CO₂. After labeling, cells were washed three times with RPMI 1640 in 37°C pre-warmed medium and resuspended in complete culture medium. For the analysis of endosomal compartments, ECs were labeled with transferrin Alexa 633, 20 μ g/ml (Molecular Probes, Leiden, The Netherlands), 30 minutes at 37°C and 5% CO₂ after DiO labeling.

For blocking experiments, exovesicles were pre-incubated 1 hour at 37°C with a blocking anti TNF- α , which is a human/mouse chimeric antibody of IgG1 κ isotope (Inflimab; Remicade). Afterward, pre-treated exovesicles were incubated with ECs for 24 hours. Supernatants were harvested and stored at -80°C until used. A panel of 14 cytokines were measured using the Luminex system from BioRad according to manufacture's recommendation. The respective human IgG1 κ was used as a control (Sigma-Aldrich, Buchs, Switzerland).

Purification of Exovesicles

Exovesicles were isolated using the standard process of a series of differential ultracentrifugation and filtration described previously.^{7,15} The supernatant of 10×10^6 DCs was collected and exovesicles were purified by centrifugation at $250 \times g$ for 8 minutes as described previously,² then run through 0.45- μ m filters to eliminate large debris. The filtered supernatant was ultracentri-

fuged at $110,000 \times g$ for 1 hour. Exovesicles were washed once with RPMI 1640 and pelleted by ultracentrifugation at $110,000 \times g$ for 1 hour. Then the pellet was resuspended in 150 μ L RPMI 1640 medium. The exovesicles were either used for immunofluorescence labeling or co-cultured with ECs.

Laser Scanning Microscopy

Laser scanning microscopy (LSM) analysis was performed with a Zeiss LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200M, Lasers: HeNe 543 nm and Ar 488 nm). Optical sections were taken with a 63x/1.4 Plan-Apochromat objective. In combination with digital zoom, this resulted in a voxel dimension of $0.1 \times 0.1 \times 0.25 \mu\text{m}$. Images were processed and visualized with IMARIS, a three-dimensional multichannel image processing software for laser scanning microscope images (Bitplane AG, Zurich, Switzerland). To quantify the intracellular exovesicles, the IsoSurface mode of the Surpass module in IMARIS was used, and an intensity threshold was applied to create a model of the data visualized as a solid surface. The intracellular objects were then counted by the software. For this quantification, all microscope settings were kept constant during one experiment, ie, for control as well as treated cultures. All settings used for the image single restoration were also equal and the cells were chosen at random. Co-localization analysis was performed with the IMARIS co-localization module. For all control stainings the same imaging parameters were used as for the specific antibodies.

Immunolabeling

DCs or exovesicles isolated from DCs unstimulated or stimulated with LPS were resuspended in complete culture medium containing 2% of alginate.¹⁶ Drops of the media were suspended carefully in a CaCl_2 solution (50 mmol/L) for 1 hour to allow the matrix formation and exovesicle immobilization. DCs or exovesicles in alginate drops were fixed in 3% paraformaldehyde containing 0.1 M/L sodium cacodylate and 7 mmol/L CaCl_2 buffer and subsequently washed in 0.1 M/L glycine, 0.1 M/L sodium cacodylate, and 7 mmol/L CaCl_2 buffer. Drops were stored in 0.1 M/L glycine, 0.1 M/L sodium cacodylate, and 7 mmol/L CaCl_2 buffer at 4°C until immunolabeling.

Cell surface markers were analyzed using pure antibodies CD83 (clone HB15a), CD40 (clone mAb89), HLA-DR (CR3/43), TNFRI (clone IP05), TNFRII (clone 22210) anti-TNF- α (MAB11), and the specific IgG1 isotype control (MOPC-21). Rhodamine-conjugated or Cy3-conjugated goat anti-mouse was used as secondary antibody (Chemicon International, Zug, Switzerland).

Protein Preparation and Electrophoresis

Proteins recovered from exovesicles were quantified with the ATTO-TAG CBQCA kit according to the manufacturer's recommendations (Molecular Probes, Leiden, The Netherlands). The fluorescence emission was mea-

sured at 550 nm (filter 530 ± 30 nm) with excitation at 465 nm (filter 485 ± 20 nm) in a CytoFluor 4000 fluorescence microplate reader (gain 40) (PerSeptive Biosystems, Foster City, CA). Sample preparation was done with 8 mol/L urea (Sigma-Aldrich Chemie BV), 2% CHAPS (Amersham Pharmacia Biotech), 20 mmol/L dithiothreitol (Sigma-Aldrich Chemie BV), 0.01% bromophenol blue (Sigma-Aldrich Chemie BV). Twelve percent SDS-polyacrylamide gel (1.0 mm 16 cm) electrophoresis was run according to manufacturer's recommendations (Protean II xi Cell; Bio Rad Laboratories, Hemel Hempstead, UK), under reducing conditions.

Western Blot Analysis

Proteins were electroblotted after one-dimensional electrophoresis onto an Immobilon P membrane (Millipore Corp.), and then incubated with a rabbit anti-human TNF- α antibody (Leinco Technologies, Inc.), followed by horseradish peroxidase-conjugated secondary antibodies. Using SuperSignal West Pico chemiluminescent substrate (Pierce Perbio Science) detection was performed on a chemiluminiscence film (Amersham, Hyperfilm). As a control, the Immobilon membrane was stripped using Restore reagent (Pierce Perbio Science) and then incubated with a mouse anti-human Actin (clone MAB1501) as previously described.

Statistics

Data are expressed as mean values with the SEM. The statistical significance was determined using Student's *t*-test. $P < 0.05$ was considered to be significant.

Results

DC-Derived Exovesicles Express MHC-II and Costimulatory Molecules Such as HLA-II, CD40, and CD83

As we have shown recently, exovesicles released from LPS-stimulated allo-DCs are able to elicit T-cell proliferation after 6 days of incubation.² Therefore, in the initial steps of these experiments, we wanted to identify the molecules that could be expressed on the surface of these exovesicles. Exovesicles in culture supernatants of LPS-activated DCs were purified using the standard ultracentrifugation and filtration process described in the Materials and Methods. DCs and isolated exovesicles were then immobilized in the alginate matrix, fixed, and immunolabeled for HLA-DR-DQ, CD40, and CD83. The fluorescence was analyzed by LSM (Figure 1). The results showed that exovesicles carried large amounts of MHC-II, CD40 and, a smaller but nonetheless clearly positive amount of CD83. These results suggest that the molecules that were already expressed on the surface of DCs (Figure 1, A, C, E) were also present on the exovesicles (Figure 1, B, D, F), thus corroborating the hypothesis that exovesicles can play a role as immuno-

Figure 1. Costimulatory molecules are expressed on exovesicles. LSM images of DCs (**A, C, E, G**) and isolated exovesicles (**B, D, F, H**) resuspended in alginate matrix and immunolabeled for the expression of HLA-DR, CD40, CD83, and isotype control. Images represent volume renderings; the control stainings and the insets in (**A, C, and E**) are single optical sections.

logical messengers of DCs, and that costimulatory molecules are present on their surface. Control cells were labeled with irrelevant IgG1 antibody resulting in no fluorescence (Figure 1, G and H).

DC-Derived Exovesicles Are Important Carriers of TNF- α

TNF- α is a pro-inflammatory cytokine and is up-regulated by LPS in DCs.^{17,18} We have therefore investigated to what extent TNF- α can be carried on these exovesicles isolated from DCs. DC-derived exovesicles, unstimulated or stimulated with 100 ng LPS, were purified using the standard ultracentrifugation and filtration process described in the Materials and Methods. Western blot analysis revealed for the first time that exovesicles from activated DCs are important carriers of TNF- α molecules, whereas under control conditions, exovesicles carry none (Figure 2A). Additionally, when we resuspended DCs in alginate previously labeled with DiO and immunolabeled with anti-TNF- α (Figure 2B), we were able to visualize a large amount of TNF- α co-localizing with the cytoplasmic membrane of immature DCs, whereas with LPS matured DCs, the internal TNF content was decreased and some TNF- α was found co-localized in

structures budding from plasma membrane. Interestingly, when we resuspended exovesicles in alginate from DCs untreated or treated with LPS immunolabeled with anti-TNFR1 and TNFR2 (Figure 2C), we were able to visualize both receptors on the membrane of the isolated exovesicles, suggesting that exovesicles are able to specifically carry TNF- α molecules on their surface when they are activated with LPS. Control exovesicles were labeled with irrelevant IgG1 antibody resulting in no fluorescence. Thus, exovesicles might play a role not only in adaptive immunity, transferring molecules involved in the activation of T-cells,² but also in innate immunity, transferring TNF- α molecules during inflammation.

DC-Derived Exovesicles Are Mostly Internalized by ECs

Recently, we demonstrated that DC-derived exovesicles are able to fuse with the plasma membrane of resting DCs in their vicinity. Therefore, we have looked to what extent DC-derived exovesicles fuse with the plasma membrane of ECs or are internalized by them. DCs were stained with the fluorescent probe DiI (red), stimulated for 10 hours with 100 ng LPS, and then exovesicles were isolated. In parallel, ECs were labeled with the DiO (green) fluorescent probes and ECs were co-cultured with exovesicles from control DCs or LPS-stimulated DCs. Cells were investigated after 1, 6, and 24 hours and the incorporation of the red fluorescence (DC-exovesicles) in green labeled cells (ECs) was analyzed (Figure 3). These exovesicles were mostly internalized by ECs, whereas in resting DCs, exovesicles mostly fused with the plasma membrane.² The amount of internalized exovesicles observed was higher in the early hours (1 hour) and decreased over 24 hours, probably due to a process of degradation and recycling of membrane proteins and lipids. At first, to find out to what extent this internalization is due to an active process, ECs were treated with 10 μ g/ml cytochalasin D, a fungal metabolite known to block phagocytosis through the depolymerization of the actin filament network. Cytochalasin D inhibited most of the internalization of exovesicles (data not shown). However, some exovesicles were still internalized and co-localization voxels were detected. These results suggest that the exovesicles might be internalized through different modes, including phagocytosis. Thus, we analyzed to what extent exovesicles are internalized through receptor-mediated endocytosis.

Exovesicles Are Partially Internalized by ECs through Receptor-Mediated Endocytosis

Internalization of exovesicles can be performed through different mechanisms, such as active phagocytosis or using receptor-mediated endocytosis, such as clathrin-coated pits, caveole, or transferrin receptors. After internalization, ligands can be recycled back to the plasma

Figure 2. Analysis of TNF- α in exovesicles. DCs were stimulated with LPS or not treated. Exovesicles were then isolated by ultracentrifugation. **A:** Western blot analysis of TNF- α in exovesicles isolated from line 1: immature DCs, line 2: LPS-activated DC-s and labeled against TNF- α (**upper panel**) or against Actin (**lower panel**). **B:** DiO-labeled DCs (green) were immunolabeled with anti-TNF- α (red). TNF- α is very abundant inside the cells (**left panel**), in contrast with LPS-DCs (**right panel**) where the cells are nearly empty of TNF- α molecules. The images represent single optical sections, the insets are volume renderings. **C:** LSM of isolated exovesicles from resting and LPS DCs resuspended in alginate matrix and immunolabeled for the expression of TNFRI (**upper panel**), TNFRII (**middle panel**), and isotype control (**lower panel**). The images represent volume renderings.

membrane or go to lysosomes or other compartments such as the Golgi apparatus, where lipids can be recycled. To determine whether exovesicles are internalized through a receptor-mediated endocytosis, isolated exovesicles from LPS-stimulated DCs or from resting DCs (labeled in red) were co-cultured with ECs labeled with DiO (green) and transferrin conjugate (blue). Cells were analyzed after 1, 3, and 6 hours and the incorporation of red fluorescence (DC-exovesicles) in green labeled ECs, or the incorporation of the red in blue endosomal compartments were analyzed using the co-localization module in IMARIS (co-localization color purple), as shown in Figure 4A. As shown previously, internalization of exovesicles was observed after 1 hour. Interestingly, some exovesicles fused with the endosomal compartments (purple), but other internalized exovesicles remained in red. When the number of co-localized voxels was analyzed (Figure 4B), an increased fusion with the endosomal compartment in a time-dependent manner was found with exovesicles originating from resting DCs (approximately 50% of internalized exovesicles), whereas endosomal fusion was reduced with exovesicles originating from LPS-DCs. These results could imply either that the turnover of exovesicles from LPS-DC is faster and exovesicles are processed and recycled or destroyed more rapidly, or that exovesicles from LPS-DCs decrease the active uptake of particles by ECs. This latter hypothesis highlights the important role of sorting during the internalization process, showing that exovesicles involved in inflammation processes are able to modulate a particular response once they are internalized.

Exovesicles from Activated LPS-DCs Induce the Release of Chemokines

To study the capacity of DC-derived exovesicles to induce a specific immune response, exovesicles were co-cultured with ECs and supernatants were harvested 24 hours later. The release of inflammatory proteins was assayed by the bioplex array system with a multiplex of 15 cytokines, chemokines, and growth factors. ECs stimulated with 100 ng LPS were used as controls. The results showed that LPS-DC-derived exovesicles are able to induce the release by ECs of major inflammatory molecules, such as IL-8, MCP-1, RANTES, MIP-1 β , TNF- α , and, to some extent, granulocyte-colony stimulating factor (G-CSF, Figure 5A). As shown in Figure 2, exovesicles are important carriers of TNF- α , and we thus asked to what extent TNF- α was involved in the stimulation of cytokine release. For these experiments, exovesicles were cultured with blocking TNF- α for 1 hour before being co-cultured with ECs. The use of TNF- α blocking antibodies, but not the control antibody, blocked the release of IL-8, MCP-1, and G-CSF, (Figure 5B). These results highlight the relevance of exovesicles as important inflammatory messengers, as they are able to activate ECs, unlike LPS alone, which did not induce the release of these cytokines by ECs (even at the high concentration of 1 μ g/ml) (Figure 5A). Soluble TNF- α is involved in the activation of IL-8, MCP-1, and G-CSF, which means that exovesicles are involved in the amplification of the innate response.

Figure 3. Incorporation of red-labeled exovesicles into green-labeled ECs. Fluorescent signals of DiO-labeled ECs (green) co-cultured with isolated exovesicles from DiI-labeled LPS-treated DCs (red) were investigated with LSM. Exovesicles from LPS-stimulated DCs are internalized by ECs, as shown in the intracellular content of ECs. An important internalization of ECs membrane can be observed co-localizing with exovesicles. Images represent xy- and xz-projections; yellow **arrowhead** marks the position of projections.

DC-Derived Exovesicles Do Not Allow ECs to Present Antigens

As shown in Figure 1, exovesicles may harbor molecules from DCs from which they originated, including MHC antigens and co-stimulatory molecules potentially involved in antigen presentation. Recently, we demon-

strated that exovesicles derived from activated dendritic cells are able to confer an antigen presentation capacity to resting DCs, which in turn activate syngeneic T-cells.

To determine the fate of exovesicles from DCs in the process of EC activation, we purified exovesicles contained in DC supernatants using the standard ultracentrifugation and filtration process described in the Materials and Methods. ECs were co-cultured with lymphocytes at a constant concentration, with or without exovesicles from allogeneic DCs, unstimulated or stimulated with LPS. As shown in Figure 6, ECs are able to induce an allogeneic activation of T-cells, as compared with T-cells alone. Although modest compared with the activation of the allogeneic DCs, which were used as the positive control in the experiment, this activation was nevertheless statistically significant. However, when DCs were added to the co-cultures of ECs and T-cells, the proliferation was significantly reduced, confirming the immunosuppressive role of the epithelium. Furthermore, when exovesicles from allo-immature dendritic cells were added to the co-culture of ECs and T-cells, instead of DCs, a systematic decrease in the proliferation of T-cells was observed when compared with the co-culture of ECs and T cells; however, this decrease was not significant. These results have shown that exovesicles are not able to transfer antigen presenting capacity to ECs, although other immunogenic properties, such as the role they can play in tolerance, require further investigation.

Discussion

Exovesicles, as well as other described microparticles and nanoparticles, such as exosomes, have raised considerable interest recently, since these microparticles were shown to transfer the function of the cell from which they originate to remote cells.^{2,19,20} One of the most important outcomes of this work is the demonstration for the first time that exovesicles from DCs are important carriers of TNF- α , and that they are involved in the acti-

Figure 4. Exovesicles from immature dendritic cells are internalized through receptor-mediated endocytosis. **A:** Volume rendering of ECs membrane DiO-labeled (green), DiI-labeled exovesicles from LPS-stimulated DCs (red) internalized by ECs, and co-localization of exovesicles in endosome compartment labeled with transferrin (purple). **B:** Diagram showing total voxel amount of the intracellular exovesicles (black bars) and co-localized voxels of exovesicles and transferrin (white bars) at 1, 3, and 6 hours. Results are expressed as means $\pm$ SEM. The asterisk represents a statistically significant difference (* $P < 0.05$) between LPS treated and control groups.

Figure 5. Exovesicles from LPS-activated DCs induce the release of chemokines by ECs. Isolated exovesicles from DCs activated with LPS or untreated DCs were co-cultured with ECs for 24 hours. Supernatants were collected and analyzed using a luminex system (Biorad). **A:** Results from six independent experiments are expressed as means $\pm$ SEM. * $P < 0.05$ between ECs stimulated with exovesicles from LPS-DCs treated and control groups. **B:** Anti-TNF- α antibodies modulate the release of IL-8, MCP-1, and to some extent, G-CSF. Isolated exovesicles from DCs activated with LPS or untreated DCs were incubated with 10 μ g/ml of anti TNF- α and co-cultured with ECs cells for 24 hours. Supernatants were collected and analyzed using a luminex system (Biorad). Results are from a typical experiment representative of five independent experiments.

vation of ECs to release additional inflammatory mediators, without conferring on ECs any antigen-presenting capacity.

Recent proteomic analyses of membrane vesicles revealed that microparticles can carry various proteins from the cells they originated from.^{21–23} However, some proteins (such as Lamp2 in exosomes) can be enriched, resulting in an individual pattern of surface molecules.^{7,24,25} The enrichment of a particular group of proteins has been associated with a particular function of the microparticles. After specific stimulation, monocyte-derived microparticles express tissue factor showing pro-coagulant properties *in vivo*.^{26,27} Microparticles from en-

dothelial progenitor cells expressing $\alpha 4\beta 1$ integrins are incorporated in endothelial cells, inducing angiogenesis through the phosphatidylinositol 3-kinase and endothelial nitric oxide synthase pathway.^{28,29} Furthermore, lymphocyte-derived microparticles have been shown to play a role in the production by monocytes of inflammatory mediators, such as TNF and IL-1 β , or of macrophage-derived microparticles, which are able to activate airway ECs.^{30,31} However, very little is known on the cytokine content of microparticles. Recently, MacKenzie and co-workers demonstrated that microvesicles derived from activated monocytes contain bioactive IL-1 β , which was able to stimulate IL-1 receptors on other cells.¹⁰ This supports our hypothesis that exovesicles might be involved in the early stages of inflammation.

We demonstrated that after stimulation with LPS, exovesicles from DCs carry large amounts of soluble 17 kDa TNF- α . TNF- α is a potent pro-inflammatory cytokine with very diverse biological activities and with important roles in various physiological and pathological phenomena. TNF mRNA can be up-regulated within 30 minutes after activation by danger signals such as LPS,³² but most TNF regulation occurs post-transcriptionally. Trimeric pro-TNF is produced after translation of TNF mRNA, which lacks the signal peptide and is inserted into the plasma membrane. Afterward, TNF is released in the extracellular milieu following the cleavage of the precursor by the TNF- α converting enzyme (TACE, ADAM 17).³³ We noticed that TNF- α can be observed underneath the plasma membrane particularly in immature DCs, but it is observed at much lower levels in LPS-DCs (Figure 2B). It is very interesting that exovesicles constitutively express both TNFRI and TNFRII molecules, but only exovesicles from activated DCs specifically carried TNF- α . Our re-

Figure 6. Analysis of antigen-presenting function of ECs by exovesicles. MLR was used to assess the transfer of alloantigen presentation by exovesicles. ECs were co-cultured with lymphocytes (Tc1) at a constant concentration (1:150) with or without exovesicles isolated from allogeneic DCs (DC2) or stimulated DCs with LPS (DC2LPS). As controls, ECs were co-cultured with T-cells and allogeneic DCs, as described in the Materials and Methods. The co-cultures were incubated for 6 days, and proliferation was measured by the incorporation of tritiated thymidine. Results from three to six independent experiments are expressed as means $\pm$ SEM. * $P < 0.05$.

sults are supported by Zhang and co-workers,³⁴ who recently have demonstrated that the TNFR1 is present in exosome-like vesicles. The authors described this result as mechanism by which soluble cytokine receptors can be generated. However, functional studies were not addressed in this paper. Based in our studies, TNF- α as well as IL1- β lack the secretory signal sequence and do not follow the classical endoplasmic reticulum-to-Golgi pathway of secretion. Therefore, exovesicles might represent an alternative mechanism of TNF transport, and stimulate specifically target cells, such as ECs.

Beside the fact that exovesicles are important carriers of TNF α , we have found that exovesicles from activated DCs are able to stimulate the release of chemokines and cytokines by ECs. Experiments blocking exovesicles with the TNF-blocking antibody inhibit the release of IL-8, MCP-1, and G-CSF, which suggests not only that exovesicles transport TNF- α , but also that the TNF- α cascade is already involved in the release of some of the inflammatory mediators of ECs. As demonstrated in Figures 3 and 4, exovesicles can be actively internalized, in part by receptor mediated endocytosis. However, as shown in Figure 4B, endocytosis in the endosomal compartments of LPS-exovesicles was reduced compared with endocytosis of exovesicles under control conditions. This might be due to the activation of ECs by biologically active exovesicles, resulting in decreased internalization.

As a mentioned in our previous article,² exovesicles were clearly detached from the DCs and were shown to be distinct from pseudopods. These exovesicles had a heterogeneous morphology and were different in size. Various sizes were observed, with small vesicles of $\sim 0.05 \mu\text{m}$, as well as larger vesicles ranging from $0.1 \mu\text{m}$ to $1 \mu\text{m}$, 90% of which ranging from $0.2 \mu\text{m}$ to $0.4 \mu\text{m}$. In the process of ultracentrifugation we cannot exclude that a fraction of exosomes may contaminate the fraction of exovesicles. However, due to fact that these exosomes from DCs are released in a minor amount compared with the exovesicles, most of the activation of epithelial cells was likely to have been conferred by the exovesicles. Interestingly, Zhang and co-workers³⁴ as well as Hawari and co-workers,³⁵ have analyzed the expression of TNFR1 in structures that they have called exosome-like vesicles, due to the fact that the TNFR1 do not co-localize with known exosome markers, suggesting that TNFR1 might be cell-type or vesicle-type specific.

Two TNF receptors (TNFR1 and 2) with low homology and different functions have been described.^{10,32} Soluble TNF- α seems to activate cells through TNFR1, which either leads to the activation of nuclear factor- κB regulating inflammatory response, or triggers stress responses resulting in apoptosis after the activation of caspase-8.³⁶ Transmembrane TNF- α seems to activate TNFR2, also leading to an inflammatory activity. Soluble TNF is able to bind to both TNFR1 and TNFR2; however, soluble TNF- α was shown to have a higher affinity to TNFR1.^{18,37} Interestingly, Legler and co-workers³⁸ demonstrated that stabilization of TNFR1 in lipid rafts plays an essential role in TNF- α mediated nuclear factor- κB activation, and consequently in reducing spontaneous apoptosis. Additionally, D'Alessio and co-workers³⁹ demonstrated that TNFR1

can be found in endosomal compartments and is even released in the membrane of exosomes.³⁵ Therefore, based on these findings and on our results, we hypothesize that exovesicles from activated DCs might activate ECs through membrane activation of raft-stabilized TNFR1 or internalization of exovesicles. The resulting release of the TNF- α content in the phagosomes or endosomes of ECs would then lead to the activation of TNFR and induce nuclear factor- κB activation, and consequently the release of cytokines, such as IL-8, MCP-1, and G-CSF. The fact that the activation of TNFR1 in the endosomal compartments can trigger apoptosis could also explain the reduced number of cells with internalized LPS-exovesicles compared with control conditions.

We have shown recently that exovesicles are released as a function of danger signals; they then fuse with the membrane of resting DCs, transferring the capacity of matured cells to immature DCs to present allo-antigen to T-cells.² Our present results have demonstrated that the microvesicular structures released from DCs carried not only MHC-II molecules, but also accessory molecules such as those allowing the activation of allogeneic T-cells. There is increasing evidence that antigen presentation is not limited to secondary lymph nodes, but can also be held in the mucosal environment.^{40,41} The airway epithelium has been shown to play an important role in the modulation of airway inflammation by the release of cytokines, chemokines, and growth factors.^{42,43} However, their role in antigen presentation is ambiguous. In a number of systems, ECs have been shown to induce tolerance and recently nitric oxide was identified as one of the major mediators for T-cell suppression by ECs.^{44–46,47} It also seems that the epithelium is able to favor the phenotype of regulatory DCs.⁴⁸ The role of ECs during renal and lung allograft rejection has been postulated.^{49–51} Cunningham and co-workers⁵² have shown that lung MHC-II-expressing ECs fail to activate allogeneic CD4 T-cells if an additional co-stimulatory signal is not provided. Thus, we asked to what extent exovesicles from DCs could transform ECs into potent APCs after the transfer of allogeneic MHC complex as well as co-stimulatory molecules. To evaluate the capacity of exovesicles to transfer antigens, exovesicles were co-cultured with ECs and the capacity to expand T-cells was measured. As shown in Figure 6, a marked suppression of T-cell proliferation was observed when DCs were co-cultured with ECs, compared with the co-culture of DCs and allogeneic T-cells. Interestingly, exovesicles were not able to transfer alloantigen-presenting capacity to ECs, suggesting that they might activate ECs to release additional immunosuppressors, such as TGF- β or nitric oxide. The immune suppression in the presence of ECs shows how important it is to perform *in vitro* experiments with co-culture systems. For instance, ECs, DCs, and macrophages have been shown to cross talk continuously *in vivo* through intercellular signaling to maintain homeostasis and coordinate immune responses.⁵³

In summary, we demonstrate that exovesicles are an important source of membrane and soluble antigens sustaining innate immunity in the airway epithelium. Exovesicles were able to activate ECs to trigger the

innate response through the release of inflammatory cytokines, but they were also able to maintain local immunosuppression.

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