Exovesicles from Human Activated Dendritic Cells Fuse with Resting Dendritic Cells, Allowing Them to Present Alloantigens

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Dendritic cells (DCs) can release microvesicles, but the latter's numbers, size, and fate are unclear. Fluorescently labeled DCs were visualized by laser-scanning microscopy. Using a Surpass algorithm, we were able to identify and quantify per cell several hundred microvesicles released from the surface of stimulated DCs. We show that most of these microvesicles are not of endocytic origin but result from budding of the plasma membrane, hence their name, exovesicle. Using a double vital staining, we show that exovesicles isolated from activated DCs can fuse with the membrane of resting DCs, thereby allowing them to present alloantigens to lymphocytes. We concluded that, within a few hours from their release, exovesicles may amplify local or distant adaptive immunological response. (Am J Pathol 2006, 169:2127–2136; DOI: 10.2353/ajpath.2006.060453)

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 providing an environmental interface, such as the skin, the respiratory system, and the gastrointestinal tract. Because of their location, immature dendritic cells (iDCs) are profoundly influenced by the environment and transmit danger signals to cells of the adaptive immune system. The presence of pathogens activates iDCs and triggers their maturation, resulting in enhanced expression of co-stimulatory molecules such as CD86 and CD80 and 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 injured tissue.¹

In the late 1980s, an alternative antigen pathway was identified as a complement to the classic pathway of lymphocyte activation. This alternative route involves, for instance, the release by follicular DCs of immune complex-coated bodies (iccosomes), thus increasing the delivery of immunogens to antigen-specific B cells in the lymph nodes.^{2,3} The various types of vesicles released into the extracellular medium from eukaryotic cells appear to have different origins: microvesicles are the result of membrane surface shedding; eg, iccosomes are produced by follicular DCs,² argosomes carry membrane-bound morphogens, as described in *Drosophila* embryos,⁴ and exosomes are the result of an exocytosis of multivesicular bodies.^{5–7} 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.^{8,9}

The shedding of membrane vesicle, or exovesicle, is thought to originate from the plasma membrane, using a mechanism similar to that of viral budding.¹⁰ Exovesicles bear most of the surface antigens expressed on the plasma membrane, with a selective enrichment in components including human leukocyte antigen class I molecules and integrins.¹¹ Their diameter ranges between ~0.1 and 1 μm , and their function has been associated to the function of the cell from which they originate.^{12,13} Many types of cells release exovesicles, but so far little is known on those originating from DCs. On the other hand, the release of exosomes by DCs has been established.^{14,15} Exosomes are defined as microvesicles of endocytic origin, cup-shaped, and ~0.05 μm in diameter. The proteins found in the membrane are mostly re-

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lated to T-cell signaling and T-cell activation, such as MHC class II, MHC class I, and CD86, but there are also adhesion molecules, such as tetraspan, and integrin proteins. The rate and relevance of the release of exovesicles versus exosomes has not yet been established.

In this study, using three-dimensional reconstructed pictures of DCs obtained by laser-scanning microscopy (LSM), we were able to identify, and quantify per cell, the secretion of microvesicles within a few hours after a danger signal such as lipopolysaccharide (LPS). These microvesicles appeared not to be of endocytic origin but seemed to be shed from the surface of DCs similar to iccosomes. Using double vital staining, we examined the interaction of exovesicles with DCs not yet activated by danger signals. We demonstrated that these exovesicles from activated DCs can fuse with the membrane of resting DCs and that they are able to transfer alloantigens to activate T cells. Our results demonstrate the origin and magnitude of the release of exovesicles by stimulated DCs potentially able to amplify even distant innate and adaptive immunity.

Materials and Methods

Monocyte Isolation and Differentiation to DCs

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,¹⁶ after spontaneous aggregation,¹⁷ and rosetting.¹⁸ In brief, 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 of streptomycin per ml, referred to as complete culture medium containing 2 μ g of polymyxin B sulfate ml^{-1} (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 gradient centrifugation. Differentiation of DCs from monocytes was performed as originally described by Sallusto and Lanzavecchia¹⁹ by culture cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng ml^{-1}) and interleukin-4 (10 ng ml^{-1}) for 6 days. The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium.

Stimulation of DCs, Co-Cultures, and Labeling

After 6 days in culture, DCs were washed and suspended at a density of 1×10^6 cells/ml in serum-free media (RPMI 1640 medium). Cells stimulated or not with 100 ng of LPS

were labeled with VIBRANT cell labeling solution DiO (3,3'-dioctadecyloxycarbocyanine perchlorate), and for co-culture conditions, either with DiO or with Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C and 5% CO_2 . After labeling, cells were washed three times with RPMI 1640 in 37°C pre-warmed media and suspended in a collagen cell culture system (Chemicom, Hofheim, Germany). Cells were incubated at 37°C in 5% CO_2 , until they were analyzed by LSM.

Exovesicle Purification

Exovesicles were isolated using the standard process of a series of differential ultracentrifugation and filtration described previously.^{20,21} DCs cultured for 6 days were 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. DCs were stimulated or not with LPS (100 ng/ml) for 24 hours. The supernatant of 10×10^6 DCs was collected, and exovesicles were purified by centrifugation at $250 \times g$ for 8 minutes as described previously²⁰ and then run through 0.22- μm filters to eliminate large debris. The filtered supernatant was ultracentrifuged 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 μl of RPMI 1640 media. The same media without cells following the same procedure of exovesicle isolation was used as control.

Lymphocyte Proliferation Assays

To perform mixed lymphocyte reactions (MLRs), 1×10^4 iDCs were co-cultured with 1×10^5 autologous lymphocytes with or without exovesicles purified from 10×10^6 autologous or heterologous pretreated DCs as described before. Co-cultures were done in triplicate in a final volume of 0.2 μl of RPMI, supplemented with 5% human serum and 2 mmol/L glutamine. The co-cultures were incubated for 6 days in a 5% CO_2 atmosphere and proliferation was measured by the incorporation of methyl-[³H]thymidine (0.5 μCi /well), which was added to the co-culture for the last 18 hours.

Laser-Scanning Microscopy

For LSM analysis, a Zeiss LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200M, lasers: HeNe 543 nm and Ar 488 nm; Carl Zeiss A.G., Feldbach, Switzerland) was used. Optical sections were taken with a 63 $\times$ /1.4 Plan-Apochromat objective. This resulted in a voxel dimension of $0.1 \times 0.1 \times 0.25 \mu\text{m}$ in combination with a digital zoom. Image processing and visualization were done using IMARIS, a three-dimensional multichannel image processing software for LSM images (Bitplane AG, Zurich, Switzerland). To quantify the released 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 cellular body and intracellular objects were removed manually before counting the number of extracellular objects. 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. The cells were chosen at random; the only criteria was that in the acquisition field an individual cell had to be present to avoid superposition of microvesicle structures from neighboring cells. Co-localization analysis was performed with the IMARIS co-localization module.

Processing of Cells for Transmission Electron Microscopy (TEM)

For TEM analysis of DCs stimulated or not with LPS, the cells were resuspended in complete culture medium containing 1.2% alginate.²² In the case of exovesicle characterization, the pellet of purified exovesicles was resuspended in complete culture medium containing 1.2% alginate. Drops of the media were suspended carefully in CaCl_2 (50 mmol/L) solution for 1 hour to allow the matrix formation and DC immobilization. DCs or exovesicles in alginate drops were fixed in 2.5% phosphate-buffered glutaraldehyde solution, postfixed in 2% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer, and contrasted in 0.5% uranyl acetate in 0.05 mol/L maleate buffer. This was followed by dehydration in a graded series of ethanol (70, 80, 96, and twice in 100%) and gradual replacement of ethanol by propylene oxide before the cells were infiltrated and embedded in epoxy resin. Ultrathin sections were cut using a Reichert Austria ultramicrotome and transferred onto 200-mesh uncoated copper grids, stained with uranyl acetate, counterstained with lead citrate according to standard methods,²³ and finally observed with a Philips 300 TEM at 60 kV (FEI Company Philips Electron Optics, Zurich, Switzerland).

Statistics

Data are expressed as mean values with the SEM. The statistical analysis was performed using SigmaStat for Windows (Version 3.10; Systat Software, Inc., or Excel for Windows) statistical software. Two groups were compared using Student's *t*-test. $P < 0.05$ was considered significant.

Results

Exovesicles from DCs Can Be Identified and Quantified by LSM

DC-secreted microvesicles were evidenced above all by transmission electron microscopy and quantified by Bradford analysis.^{24–26} To determine the dynamics of exovesicle release as well as identify their origin, we analyzed the release and quantification of exovesicles

from blood monocyte-derived DCs. The cells were stained with DiO, a lipophilic fluorescent green probe, which is incorporated into the membrane lipid bilayers of living cells. The cells were cultured in a collagen matrix to immobilize the cells and visualize the released vesicles after LPS stimulation.

Cell image acquisition was done by LSM. To improve the visualization of the three-dimensional data set, shadow projection (IMARIS) from top (Figure 1A) or volume rendering from the side (Figure 1B) was performed. However, these visualization modes have not yet provided an identification of objects released from the cell body. Therefore, the cells were further analyzed using the IsoSurface mode of the Surpass module in IMARIS by applying a surface algorithm (Figure 1C). The object was then segmented into individual objects, which separated the cell body from external objects, ie, the microvesicular structures (Figure 1D). After removing the cell body as well as intracellular objects, the external objects could be visualized (Figure 1E). 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. Precise confocal data could be used for the analysis of these exovesicles, particularly for their quantification.

DC Exovesicle Release Is Dependent on Danger Signals Such as LPS

The number of exovesicles released after 2, 6, and 24 hours in the matrix with or without LPS stimulation was quantified. In Figure 2A, exovesicles are shown surrounding the cells. In control cultures, the number of external objects increased transiently in a time-dependent manner, and the number increased up to three to four times when cells had been activated with LPS. The data are summarized in Figure 2B. Whereas iDCs released spontaneously 45 (SD, 25) to 120 (SD, 81) exovesicles per cell, the number of vesicles released by LPS-stimulated DCs was much higher, up to 247 (SD, 125) per cell after 24 hours of stimulation, with a peak value of 362 (SD, 128) at 6 hours. The diameter of these particles was between 0.1 and 5 μm , and 90% of them had a diameter between 0.2 and 0.4 μm (Figure 2C). No significant difference in size was observed between unstimulated or LPS-stimulated in an early (6 hours) or late (24 hours) release exovesicles. This finding suggests that DCs are able to respond to danger signals by releasing exovesicles of different sizes, including those consistent with an endosomal origin (exosomes from 0.05 to 0.09 μm), although most of them are larger exovesicles probably budding from the plasma membrane.

DCs Release Mostly Microvesicles Budding from the Cell Surface

TEM micrographs of DCs immobilized in alginate matrix display cells with the absence of apoptotic features such as DNA fragmentation or chromatin condensation and

Figure 1. Visualization of individual objects by combining LSM and advanced digital image restoration. Cells were cultured in a collagen matrix to immobilize the cells and released vesicles after LPS stimulation for 24 hours. All images represent the same confocal data set taken from one cell. **A:** Three-dimensional reconstruction from top; **B:** volume rendering from the side; **C–E:** surface rendering from the cell (green), segmented into individual objects (cell body yellow, external objects turquoise).

small spherical bodies (Figure 3A) surrounding cells. These microvesicles appear budding from the plasma membrane and to lay free, separated from the cells (inset A' and A'', arrows). After isolation of exovesicles by ultracentrifugation, the pellet was resuspended in the alginate matrix. We observed some of these exovesicles trapped in the matrix (Figure 3B) with different shapes. These exovesicles contained no prominent organelles and had a lipid bilayer cell membrane. We observed some material released by endosomal exocytosis, which might be related to the release of exosomes previously described.^{25,27} However, this form of microvesicles appeared to be insignificant. These results suggest that DCs are able to release many microvesicles, most of them budding from the surface or from the elongated processes of DCs. We consider this process as an exovesicle production, identical to that previously described.^{12,13}

Exovesicles Released from Stimulated DCs Can Fuse with the Cytoplasmic Membrane of Resting DCs in Co-Cultures

To test the ability of exovesicles to fuse with resting DCs in their vicinity, DCs were stimulated for 12 hours with 100 ng of LPS and stained with the fluorescent probe DiI (red) (LPS-DC). Prelabeled iDCs with the DiO (green) fluorescent probes were co-cultured with LPS-DCs in a collagen matrix. Cells were investigated after 6 and 24 hours, and the incorporation of the red fluorescence (LPS-DCs) in green labeled cells (iDCs) related to the cell volume was analyzed using the co-localization module in IMARIS.

When iDCs were co-cultured with control cells (no LPS stimulation), only a small amount of incorporated red material could be seen in green labeled cells, even if the cells were in close contact, as shown in Figure 4A. Con-

Figure 2. Visualization and quantification of released exovesicles. **A:** LSM images of iDC compared with LPS-matured DCs after 2 (**a, d**), 6 (**b, e**), and 24 (**c, f**) hours. iDCs (**a–c**) and LPS-DCs (**d–f**), in collagen matrix, showing the release of exovesicles (turquoise vesicles) at different time points. **B:** Total amount of exovesicles released by DCs, calculated by the IMARIS software at the different time points, ie, 2, 6, and 24 hours. Data are expressed as mean $\pm$ SD of two experiments with scanning of 8 to 10 cells each by LSM. The **asterisk** represents a statistically significant difference ($P < 0.01$) between LPS-treated and control groups. **C:** Size frequency of exovesicles, calculated by the Surpass module software in IMARIS, of DCs and LPS-stimulated DCs at 6 and 24 hours in the co-culture conditions. Bars are means $\pm$ SD of nine data sets.

versely, when iDCs were co-cultured with stimulated LPS-DCs, at an early time (6 hours), nonstimulated DCs incorporated labeled vesicles into their membrane, as shown by the incorporation of red material (Figure 4B). The number of co-localized voxels was analyzed. It resulted in 15.2 (SD, 9.4) co-localized voxels per μm^3 for iDC co-cultures with LPS-DCs, compared with 5.2 (SD, 5.5) co-localized voxels per μm^3 in control co-cultures (Figure 4B). The number of co-localized voxels decreased after 24 hours and was 4.2 (SD, 5.3) voxels per μm^3 for iDC co-cultures with LPS-DCs, compared with 1.4 (SD, 3.1) voxels per μm^3 in control cultures (Figure 4B). The rate of internalization of DC exovesicles was low compared with the fusion (Figure 4B), which suggests that exovesicles lodged within the cell surface membrane probably play a role in antigen presentation.

LPS-DCs Co-Cultured with Resting DCs Induce the Release of Exovesicles by Resting DCs

We examined the ability of iDCs to release exovesicles at 6 and 24 hours in the vicinity of DCs previously activated for 12 hours by LPS and washed or in contact with control DCs. Figure 5A shows one activated DC in co-culture for 24 hours with a nonactivated iDC. The iDC has incorporated red material (Figure 5A'). The release of external objects from such nonactivated iDCs which had fused with exovesicles was analyzed as described for directly stimulated DCs. Our results show that nonstimulated co-cultured DCs were able to release exovesicles very early on (Figure 5B), with a mean of 7 (SD, 4) exovesicles during the first 6 hours and 88 (SD, 84) exovesicles after

24 hours. When resting DCs were co-cultured with LPS-matured DCs, the number of exovesicles increased from 21 (SD, 18) at 6 hours to 442 (SD, 276) at 24 hours. This suggests that, even though they were not directly stimulated with LPS, resting iDCs were able to sense a danger signal by fusing LPS-DCs exovesicles within their own membrane. They were then able to respond themselves by releasing a new burst of exovesicles, whereas LPS-DCs, after 24 hours, produced only very small numbers.

Exovesicles from DCs Confer Alloantigen Presenting Capacity

Exovesicles may harbor molecules from the DCs from which they originated, among which there may be major histocompatibility antigens and co-stimulatory molecules.⁷ These exovesicles may thus confer antigen presentation capacity to the DCs with which they fused. To determine the functional role of these exovesicles released from DCs, exovesicles contained in culture supernatants of LPS-activated or nonactivated DCs were purified using the standard ultracentrifugation and filtration process described in Materials and Methods. For the MLR, DCs were co-cultured with syngeneic lymphocytes at a constant concentration with or without exovesicles from syngeneic or allogeneic DCs. Exovesicles from LPS-stimulated allo-iDCs were able to elicit T-cell proliferation after 6 days of incubation (Figure 6). In five independent experiments, the intensity of T-cell stimulation was 3.5-times increased with exovesicles derived from allo-LPS-DCs than with exovesicles derived from allo-iDCs with a respective proliferation of 30,178 cpm (SEM $\pm$ 13,273)

Figure 3. TEM of human DCs processed using alginate matrix. **A:** LPS-DCs cultivated for 6 hours in alginate matrix showing cell bodies and ruffling of the plasma membrane. In our TEM analysis, we confirm the absence of extensive apoptosis or apoptotic bodies. Insets **A'** and **A''** show exovesicle (**arrows**) close to the cell. Inset **A'** shows details of exovesicle budding (**arrowhead**). **B:** Characterization of LPS-DC-derived exovesicles. Exovesicles from 10×10^6 LPS-DCs obtained after filtration and ultracentrifugation were resuspended in alginate. Different shapes of exovesicles are seen with a bilayer lipid membrane and suspended in the matrix.

compared with 8759 cpm (SEM ± 4573) in the control condition ($P < 0.05$). In the experiment in which exovesicles derived from LPS-DCs were co-cultured with

allogeneic lymphocytes without APCs, there was no significant induction of T-cell proliferation, indicating that DCs were required to induce alloantigen proliferation of T

Figure 4. Co-localization analysis of the incorporation of red-labeled exovesicles into green-labeled cells. Three-dimensional data of DiO-labeled iDCs (green) co-cultured with DiI-labeled LPS-treated DCs (red) in collagen matrix were taken with LSM and co-localization analysis was performed. **A:** Co-cultures of control cells (red and green). No red signal is seen in green cells. **B:** Exovesicles released from LPS-stimulated DCs fuse with the plasma membrane of iDCs, as shown by the incorporation of red material (yellow indicates co-localization of green and red, **arrows**). Little intracellular red material is seen (**arrowhead**). Images represent xy - and xz -projections; **yellow arrowheads** mark the position of projections. Insets represent three-dimensional reconstructions from the same data sets. **C:** Quantification of co-localized voxels related to the volume (μm^3) in the plasma membrane of iDCs in the co-culture of nonstimulated DCs (iDC DiO – iDC DiI) or in the co-culture with LPS-stimulated DCs (iDC DiO – LPS-DC DiI). Data are expressed as mean $\pm$ SD of three experiments with LSM scanning of 10 cells each. The **asterisk** represents a statistically significant difference ($P < 0.02$) between LPS-treated and control groups.

cells. In two control experiments, autologous MLR presentation (AMLR) was observed when syngeneic DCs and T cells were also incubated with syngeneic exovesicles from LPS-activated DCs. However, this AMLR reached 55% proliferation induced by allogeneic vesicles derived from LPS-stimulated DCs. One representative experiment with all controls is shown in Figure 6.

Discussion

The major outcome of our work was to visualize and quantify for the first time the amount of exovesicles released per DC, particularly on LPS stimulation, using LSM in combination with advanced image restoration. With the Surpass module in IMARIS, we were able to quantify up to 900 exovesicles around stimulated cells. Various sizes

were observed, with small vesicles of $\sim 0.05 \mu\text{m}$ as well as larger vesicles ranging from 0.1 to $1 \mu\text{m}$, 90% of which ranging from 0.2 to $0.4 \mu\text{m}$. However, there was no difference in size between vesicles released after 6 hours and after 24 hours of LPS or control conditions. Despite the fact that a variety of shapes was observed, the proportion of microvesicles with a size similar to that of exosomes (0.05 to $0.09 \mu\text{m}$) was low (less than 5%), suggesting that exosomes could be released in smaller amounts than exovesicles. After labeling the cell membrane with DiO tracer, internalization of the labeled plasma membrane to form endosomes and then multivesicular endosomes was observed only occasionally. Therefore, we hypothesized that at least 90% of the microvesicles released were shed from the plasma membrane.

Figure 5. Release of exovesicles from iDCs in the vicinity of mDCs. **A:** LSM image of DiO-labeled iDCs (green) co-cultured with DiI-labeled LPS-stimulated DCs (red) in collagen matrix for 24 hours. In **A'**, incorporated red material can be observed (arrows). **A:** Three-dimensional reconstruction; **A':** xy- and xz-projections from the same data set; yellow arrowheads mark the position of projections. **B:** Diagram showing total amount of external objects released by iDCs in the co-culture of nonstimulated DCs (left panels) or in the co-culture with LPS-stimulated DCs (right panels). Data are expressed as mean $\pm$ SD of two experiments with LSM scanning of 8 to 10 cells each. The asterisk represents a statistically significant difference ($P < 0.001$) between LPS-treated and control groups.

TEM was used to morphologically characterize DCs by the presence of elongated processes. As shown in Figure 3, DCs were able to release microvesicles of various shapes and sizes, with a single membrane and a homogeneous content. These exovesicles visualized by TEM originated from cells that did not have a dense nucleus, typical of cells undergoing apoptosis, and they were very few structures consistent with apoptotic bodies ($<2\%$) of ~ 1 to $4 \mu\text{m}$ in size.²⁸

Nonactivated DCs release exovesicles in a process similar to the release of exosomes described in the literature.¹⁵ This is demonstrated by the constant increase in numbers released from an early time (2 hours) up to 24 hours. However, when DCs are activated, the release increases early on (2 hours) before reaching a peak at 6 hours (Figure 2B). If we correlate these time points with the different stages of DC life, ie, recruitment, antigen

uptake, and initial migration to lymph nodes, those exovesicles might be deemed to play a particular role in innate immune response and initial inflammation. Recently, MacKenzie and co-workers²⁹ demonstrated that microvesicles from activated monocytes contain bioactive IL-1 β , which was able to stimulate IL-1 receptors on other cells. This supports our hypothesis that exovesicles might be involved in the early stages of inflammation.

Our finding that the number of exovesicles released from activated DCs is higher than that produced by resting DCs may contradict the results from other authors,^{24,30} who reported that the release of what they name exosomes decreases on maturation of DCs (mDCs), and that mDCs consistently secrete approximately two to three times less exosomes than iDCs, probably attributable to a reduction of endosomal activity during maturation.^{15,24} However, besides the total

Figure 6. Analysis of antigen-presenting function of DCs by exovesicles. MLR was used to assess the stimulatory function of exovesicles. DCs (DC1) were co-cultured with syngeneic lymphocytes (T1) at a constant concentration with or without exovesicles isolated from allogeneic pretreated DCs (DC2 and DC2-LPS) or syngeneic pretreated DCs (DC1 and DC1-LPS) as controls, as described in Materials and Methods. DC1/T1 cell ratio was 1:10. The co-cultures were incubated for 6 days and proliferation was measured by the incorporation of tritiated thymidine. One of five independent experiments, done in triplicate, is shown. Results are expressed as means $\pm$ SD. One **asterisk** represents a statistically significant difference ($P < 0.01$) compared with T1 + DC1 + Exo (DC2) control conditions, and two **asterisks** represents a statistically significant difference ($P < 0.05$) compared with T1 + DC1 + Exo (DC1-LPS) control condition.

amount released significantly higher at all time points with LPS-DCs than with iDCs, we observed special kinetics of the exovesicle release from LPS-DCs. The release peaked at 6 hours and decreased at 24 hours, showing that exovesicles as was demonstrated by exosomes²⁴ can be developmentally regulated as a function of the maturation of DCs.

DCs were immobilized in a three-dimensional cell collagen culture system. In the co-culture system, we observed that microvesicular structures released from activated DCs can migrate and fuse with the membrane of resting DCs. This phenomenon was measured by counting the number of voxels in the membrane of resting DCs, which was high early on (6 hours) and decreased at 24 hours. This decrease may reflect the amount of vesicles available or a reduced capacity to integrate external material, which can be correlated with the maturation process of DCs fusing with microvesicles. Indeed, once the cells have integrated the exovesicles, they may be activated and lose some of their absorption capacity³¹ and enhance the turnover of their membrane component, thereby decreasing the visibility of the fused microvesicles on their surface. In addition, at the same time point, this matured cell increased its release of exovesicles as shown in Figure 5B. This supports the idea that exovesicles play a role as immunological messengers leading to the maturation/activation of neighboring DCs. This property could be linked with soluble or membrane-bound mediators as previously suggested²⁹ or to the release of cytoplasmic components in the cells with which microvesicles did fuse.

The ability to prime naive T cells constitutes a unique and critical function of DCs. Despite this, exosomes have been shown to mediate transfer of membrane material between different cells, but it is not clear whether these

microvesicles modulate T-cell tolerance or priming. Internalization of microvesicles has been demonstrated with a transfer of functional MHC class I to acceptor DCs for presentation to CD8⁺ T cells.³² In addition, exosomes from iDCs or mDCs display different qualitative protein composition.¹⁵

In our experiments, exovesicles released by allogeneic LPS-DCs transferred to resting DCs the capacity to prime T cells. We have shown that exovesicles were released as a function of danger signals; they could fuse with the membrane of resting DCs, transferring the capacity of matured cells to iDCs to present alloantigen to T cells. The microvesicular structures fused with iDCs might play a role in the activation of resting DCs but imply also the transfer of allo-MHC molecules and perhaps accessory molecules to allow MLR to be induced. Indeed, exovesicles isolated from LPS-stimulated autologous DCs are able to increase AMLR but at a rate still significantly lower than exovesicles isolated from allogeneic DCs, reflecting that this process is not a carryover of LPS but an increase of MHC on the DC surface. However, exovesicles released spontaneously by resting allogeneic or syngeneic DCs do not activate T cells. Our results corroborate the results of other authors, in which quiescent DCs were shown to help maintain a state of peripheral T-cell tolerance, as demonstrated by Shortman and Liu,³³ thereby showing that exovesicles from resting DCs might contribute to tolerance. Furthermore, our experiments corroborate the fact that those vesicles do not present alloantigens to activate T cells but required the presence of DCs,³⁴ thereby supporting the idea that these exovesicles are functionally very similar to exosomes and alone do not support T-cell proliferation.

In summary, the data generated by LSM and TEM provide new insights in the release of exovesicles from DCs. Overall, DCs are able to release numerous exovesicles from their plasma membrane in response to danger signals, a mechanism that appears to be much more prominent than the release of exosomes from multivesicular endosomes. Those exovesicles released from activated cells were integrated in the membrane of adjacent resting DCs, which in turn induced an activation and release of exovesicles from the plasma membrane. These exovesicles could transfer MHC molecules and antigens such as alloantigens to be presented by neighboring DCs to T cells.

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