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Extracellular Vesicles with Exosome-like Features Transfer TLRs between Dendritic Cells

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ABSTRACT
Accumulating evidence shows that extracellular vesicles (EVs) secreted by immune cells play an important role in intercellular communication. In the current report, we show that EVs released from wild-type bone marrow–derived dendritic cells (BMDCs) transfer TLRs to TLR4-knockout (TLR4KO) BMDCs and increase cellular responsiveness to LPS in recipient cells. The transferred EVs have exosomal characteristics and induce the activation of NF-κB signaling pathways in recipient cells. We further show that BMDC-derived EVs can promote LPS-induced inflammation in TLR4KO mice in vivo. These results indicate that functional TLR4 can be transferred from wild-type to TLR4KO BMDCs through exosome-like EVs. ImmunoHorizons, 2019, 3: 186–193.

INTRODUCTION
Dendritic cells (DCs) are a class of professional APCs that express multiple types of TLRs (1). Within the family of TLRs, TLR4 is a cell membrane receptor that recognizes LPS, which is a major component of the outer membrane of Gram-negative bacteria (2). Upon ligand binding, TLR4 activates a set of adaptor proteins and intracellular kinases, leading to the nuclear translocation of transcription factor NF-κB and the production of proinflammatory cytokines such as IL-6 and TNF-α (3). TLR4 activation results in enhanced inflammatory responses that are positively associated with a variety of immune diseases, such as arthritis (4) and inflammatory bowel disease (5), and it has also been shown to be involved in mechanisms associated with immune therapy in cancer patients (6).

Recent studies have revealed that exosomes, which are extracellular vesicles (EVs) that are 30–100 nm in size that originate from endosomes, could be effective mediators of intercellular communication between local immune systems. Exosomes derived from DCs are known to stimulate CD4+ T cells and elicit specific immune responses (7). Additionally, it has been reported that exosomes derived from bone marrow–derived DCs (BMDCs) incubated with rIL-10 can increase the number of regulatory T cells in the intestinal mucosa and enhance protective effects against colitis in a mouse model (8). However, the mechanisms underlying the immune regulatory effects exerted by exosomes have not yet been completely elucidated.

EVs contain a variety of biological components, including membrane proteins, lipids, microRNA (miRNA), mRNA, and DNA. Previous studies demonstrated that DC-derived exosomes could exert biological effects via the functional transfer of sorted miRNAs (9). However, few studies have shown that proteins within exosomes can be transferred or induce functional changes in recipient cells (10). In this study, we show for the first time, to our knowledge, that TLR4s on BMDC-derived EVs that have exosome-like features can be taken up by other DCs and activate the TLR4/NF-κB signaling pathway in recipient cells.
MATERIALS AND METHODS

Animals
Eight-week-old C57BL/6J (wild-type [WT]) and C57BL/10ScNCR (TLR4-knockout [TLR4KO]) mice were purchased from The Jackson Laboratory (https://www.jax.org/strain/003752; Bar Harbor, ME). Animal maintenance and care procedures were conducted according to the policies of the Institutional Animal Care and Use Committee at the University of Miami.

WT and TLR4KO mice received tail-vein injections of 1 mg (total protein) of exosomes in PBS. Twenty-four hours later, the mice received 500 ng/kg LPS by i.p. injection. Two hours after this, the mice were sacrificed, and their serum, spleen, and mesenteric lymph nodes (MLN) were processed for cytokine measurement.

Primary DC culture
WT and TLR4KO mice were sacrificed, and their femur bones were removed for bone marrow primary culture. The bone marrow cells were maintained in complete IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS, 1% penicillin/streptomycin, 20 ng/ml GM-CSF, and 10 ng/ml recombinant mouse IL-4 protein (R&D Systems, Minneapolis, MN). BMDCs were generated by 10–12 d of differentiation in complete medium.

Flow cytometric analysis
BMDCs were stained for DC markers according to a method described in previous studies (11). Forward and side scatter gating were used to remove debris while preserving singlet events. The panel of Abs used in these experiments included CD11b-conjugated Alexa Fluor 488 and CD11c-conjugated eFluor 660, which were obtained from eBioscience, and TLR4–BV421 and CD45–redFluor 710, which are obtained from BD Biosciences. Flow cytometry was performed using a CytoFLEX flow cytometer.
Electron microscopy

EVs were recovered from the supernatant of BMDCs cultured in medium depleted of FBS-derived exosomes. The supernatant was centrifuged at 3000 \( \times g \) for 10 min to remove the cell debris and was then purified by filtration with 0.20-\( \mu \)m pore filters, followed by ultracentrifugation at 100,000 \( \times g \) at 4\(^\circ\)C for 2.5 h as previously described (12) or isolation using an ExoQuick isolation kit (System Biosciences, Palo Alto, CA) based on the manufacturer’s protocol.

The isolated EVs were fixed in 2% paraformaldehyde, and 10 \( \mu l \) of each sample was loaded onto a Formvar/carbon-coated copper grid for 30 min. The grid was rinsed by floating it on top of a drop of 0.1 M PO\(_4\) buffer for 5 min, and then the EVs were fixed in 2% glutaraldehyde fixative for 5 min; the grid was washed in 0.1 M PO\(_4\) buffer for 5 min and then in double-distilled water for 5 min, after which the procedure was repeated. The grid was then floated on top of a drop of 4% uranyl acetate in double-distilled water for 5 min in the dark. The grid was drained and completely dried in the dark and then transferred to a grid box. Images were acquired with a JEOL JEM-1400 Transmission Electron Microscope (Peabody, MA) equipped with a Gatan Orius SC200D digital camera. The University of Miami transmission electron microscopy (TEM) imaging core acquired all TEM images of the EVs.

Western blot analysis

Total protein (20 \( \mu g \)) was extracted from BMDCs or exosome pellets lysed with radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA) containing fresh protease and phosphatase inhibitors (Roche, Indianapolis, IN) and was then loaded into each lane and separated by NaDodSO\(_4\) PAGE. The proteins were then transferred to a 0.45-\( \mu \)m pore-size nitrocellulose membrane (Bio-Rad, Hercules, CA). The Ab used in the Western blot assay is shown in Table I.

Immunocytochemistry

The EVs were labeled with a lipid-associating fluorescent dye (PKH67; Sigma-Aldrich, St. Louis, MO). BMDCs were grown in a Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). Fluorescently labeled exosomes or PBS–PKH67 controls were washed with PBS and incubated with the cells for 24 h. On the following day, the cells were washed twice with PBS, fixed in 4% paraformaldehyde, and stained with Alexa Fluor 594 Phalloidin.

Figure 3. BMDCs from TLR4KO mice gained responsiveness to LPS after the uptake of exosomes from WT cells. (A and B) In TLR4KO BMDCs, treatment with LPS does not stimulate cytokine production. After incubation of exosomes from WT BMDCs and LPS stimulation for 4 h, real-time PCR showed that TNF-\( \alpha \) and IL-6 mRNA levels were significantly increased. (C and D) In the supernatant of WT BMDCs, LPS stimulated a high level of TNF-\( \alpha \) and IL-6 production. In TLR4KO BMDCs, only preincubation with exosomes from WT BMDCs and treatment with LPS could stimulate the production of the proinflammatory cytokines TNF-\( \alpha \) and IL-6, as measured by ELISA. The results are expressed as the mean ± SEM of triplicate measurements in each group. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \). Exo(wt/LPS), exosomes purified from the supernatant of WT BMDCs pretreated with LPS; Exo(wt/S), exosomes purified from the supernatant of WT BMDCs pretreated with saline; sal, saline.
(Thermo Fisher Scientific) for 60 min. The sample slides were washed with PBS and mounted with DAPI antifade reagent. Images were obtained using a Leica microscope.

Quantitative real-time PCR

Total RNA was isolated from exosomes or BMDCs with TRIzol reagent (Invitrogen, Carlsbad, CA). The levels of IL-6, TNF-α, and TLR4 were detected by quantitative real-time PCR using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and a LightCycler 480 System (Roche). The primers used for quantitative real-time PCR are shown in Table II.

ELISA

Exosomes (100 ng of total protein) were added to BMDCs and cultured in bovine exosome-free medium for 24 h. The conditioned medium was collected and centrifuged (3000 × g) to clear the supernatants, which were subsequently used for cytokine detection by ELISA. For the in vivo experiments, serum, spleen, and MLN extracts from both WT and TLR4KO mice were centrifuged, and the total protein concentration in the supernatant was measured for the purpose of normalization. The cytokines in the cell culture supernatant or in animal organs were detected by TNF-α and IL-6 ELISA kits (Invitrogen Life Technologies) according to the manufacturer’s protocols. The cytokine concentrations were determined by the measurement of absorbance in triplicate with a SpectraMax M5 plate reader and SoftMax Pro 5 software.

Statistics

Data are presented as the mean ± SEM. The statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Unpaired Student t tests or one-way ANOVA was used for the analysis. The significance levels were denoted as follows: *p < 0.05, **p < 0.01, or ***p < 0.001.
RESULTS

DCs secrete and take up EVs with exosomal features

To validate the primary culture, CD45/CD11b/CD11c and TLR4 Abs were used as cell surface markers for the flow cytometry assay. Our results show that a significant population within the bone marrow cells had differentiated into DCs in 10–12 d (Supplemental Fig. 1), and there was a distinct difference in TLR4 expression in WT and TLR4KO BMDCs (Supplemental Fig. 2). Two methods were used to purify the EVs: ultracentrifugation or isolation with an ExoQuick Kit from System Biosciences. The EVs that were harvested from the supernatants of BMDC cultures were visualized by TEM. In both samples, we observed round-shaped vesicles between 20 and 100 nm in diameter. The particle size distribution of these receptors by LPS-induced activation. Western blot analysis of the cytosolic and nuclear proteins from BMDCs showed that, after the preincubation of WT exosomes and stimulation by LPS for 4 h (Fig. 4A), the IκBα protein level in the cytosol of the TLR4KO BMDCs decreased (Fig. 4B) and NF-κB protein translocated from the cytoplasm (Fig. 4C) to the nucleus (Fig. 4D), both of which indicate the activation of the NF-κB signaling pathway. These results also provided evidence that exosomes transferred TLR4 between DCs as cargo. The activation of these receptors led to activation of the NF-κB intracellular signaling pathway and the production of inflammatory cytokines in the presence of the TLR cognate ligand LPS (Tables I, II).

TLR4KO mice responded to LPS following i.v. injection of exosomes from WT BMDCs

To further confirm the effect of exosomal TLR4 transportation in vivo, we administered exosomes from WT or TLR4KO BMDCs to TLR4KO mice by tail-vein injection. Twenty-four hours after PBS or exosome injection, the WT or TLR4 mice were injected with LPS i.p. Two hours later, the serum, spleen, and MLN of these mice were harvested for the analysis of proinflammatory cytokine production (Fig. 3C, 3D). Only the addition of LPS to recipient cells in the presence of exosomes from WT BMDCs could induce the TLR4KO BMDCs to become responsive to LPS, suggesting that functional TLR4s were transferred from the WT exosomes to the TLR4KO cells.

<table>
<thead>
<tr>
<th>TABLE I. Primary Abs used in Western blot assay</th>
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<tbody>
<tr>
<td><strong>Ab</strong></td>
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</tr>
<tr>
<td>CD63 (H-193)</td>
</tr>
<tr>
<td>CD 9 (EM-04)</td>
</tr>
<tr>
<td>Tsg101</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td>IκBα</td>
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<tr>
<td>NF-κB (p105/p50)</td>
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<td>TBP</td>
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Determined that recipient cells with F-actin (red) or IL-6, TNF-α, or IL-6 mRNA between these exosome samples (Supplemental Fig. 3C). Additionally, exosomes purified from the supernatants of WT BMDCs that were pretreated with LPS alone did not stimulate TLR4KO BMDCs to produce proinflammatory cytokines (Fig. 3C, 3D). Only the addition of LPS to recipient cells in the presence of exosomes from WT BMDCs could induce the TLR4KO BMDCs to become responsive to LPS, suggesting that functional TLR4s were transferred from the WT exosomes to the TLR4KO cells.

The NF-κB signaling pathway was activated in TLR4KO BMDCs upon exosomal uptake from WT cells

Because we observed the uptake of TLR4s from WT exosomes by TLR4KO BMDCs, we next investigated the direct signaling targets of these receptors by LPS-induced activation. Western blot analysis of the cytosolic and nuclear proteins from BMDCs showed that, after the preincubation of WT exosomes and stimulation by LPS for 4 h (Fig. 4A), the IκBα protein level in the cytosol of the TLR4KO BMDCs decreased (Fig. 4B) and NF-κB protein translocated from the cytoplasm (Fig. 4C) to the nucleus (Fig. 4D), both of which indicate the activation of the NF-κB signaling pathway. These results also provided evidence that exosomes transferred TLR4 between DCs as cargo. The activation of these receptors led to activation of the NF-κB intracellular signaling pathway and the production of inflammatory cytokines in the presence of the TLR cognate ligand LPS (Tables I, II).

<table>
<thead>
<tr>
<th>TABLE II. Primers used in real-time PCR assays</th>
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<tr>
<td><strong>Genes</strong></td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>IL-6 F</td>
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<tr>
<td>IL-6 R</td>
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<tr>
<td>IL-1B F</td>
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<tr>
<td>IL-1B R</td>
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<tr>
<td>TNF-α F</td>
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<td>TNF-α R</td>
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<tr>
<td>TLR4 F</td>
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<tr>
<td>TLR4 R</td>
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<tr>
<td>GAPDH F</td>
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<tr>
<td>GAPDH R</td>
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<tr>
<td>rpl32A F</td>
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<td>rpl32A R</td>
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F, forward primer; R, reverse primer; rpl32A, 60S ribosomal protein L32 A.
animals were collected for the detection of IL-6 and TNF-α by ELISA. WT mice, but not TLR4KO mice, responded to LPS stimulation (Fig. 5). However, TLR4KO mice injected with exosomes from WT BMDCs showed significant responsiveness to LPS, as indicated by IL-6 and TNF-α production in serum (Fig. 5A–D), spleen (Fig. 5E–H), and MLN (Fig. 5I–L). TLR4KO mice injected with exosomes from TLR4KO BMDCs did not show similar changes in immunomodulatory efficacy, indicating that exosomes alone do not contribute to this effect.

In addition, we found that the transfer of exosomes derived from WT BMDCs to TLR2-knockout BMDCs induced TLR2-knockout BMDCs to respond to denatured Enterococcus faecalis. Interestingly, these BMDCs produced IL-6 but not TNF-α poststimulation (Supplemental Fig. 4). Overall, these observations support our hypothesis that functional TLR4 and TLR2 proteins can be transferred via exosomes between cells.

**DISCUSSION**

In this study, we used multiple approaches to confirm that exosomes derived from WT BMDCs transferred TLR4 to TLR4KO cells and altered the ability of the recipient cells to respond to LPS stimulation both in vitro and in vivo. To determine whether the TLR4 membrane protein was taken up by the recipient cells and if it triggered the inflammatory responses in the TLR4KO cells, different controls were used. Exosomes from WT BMDCs alone did not induce the release of proinflammatory cytokines in the recipient cells, even when the donor cells were pretreated with LPS, indicating that only the TLR4, but not the downstream signaling molecules in the TLR4/NF-κB pathway, such as activated myd88, TRAF6, or IRAK4, were transferred via exosomes to the TLR4KO cells (Fig. 3). In addition, the difference in the immunomodulatory characteristics of the recipient cells is not
due to the transfer by exosomes of mRNA encoding the cytokines being investigated (Supplemental Fig. 3C). The comparison between TLR4KO and WT exosomes in vivo further confirmed that functional TLR4 protein in the exosomes is required for the recipient cells to respond to LPS (Fig. 5). In addition, we are able to address the possibility that other membrane-associated proteins besides TLR4, such as TLR2, can also be transferred to other cells via exosomes (Supplemental Fig. 4).

There are several mechanisms underlying the amplification of inflammatory responses to LPS in TLR4KO BMDCs. For instance, it is plausible that WT exosomes shuttle nucleic acids (mRNA or miRNA) between WT and knockout cells and regulate functioning of the recipient cell at a posttranscriptional level, and many studies have demonstrated that this functional change is correlated with exosomes (12, 13). However, we believe that the most likely mechanism involves the fusion of the exosomal membrane with the recipient cell membrane to transfer the TLR4 binding moiety to the recipient cell, because most other signaling molecules are still intact in the TLR4/2-knockout DCs. This mechanism of transfer is supported by studies that show that the lipid bilayer of the exosome, in addition to protecting the exosomal cargo from plasma and immune components, is also instrumental in delivering exosomal cargo and exosomal membrane components from donor cells to recipient cells by endocytosis or fusion without compromising the intrinsic functioning of the membrane or cargo (14, 15).

There is growing interest in the therapeutic uses of exosomes, both in inflammatory diseases and in cancer therapies. The small size of exosomes distributed in biofluids such as serum and milk allows them to travel long distances in the human body (16). Exosomes could be used as mediators for the exchange of bioinformation between neighboring cells and the maintenance of the homeostatic microenvironment during immune responses (9) or as stable carriers for the delivery of RNA/protein content to a defined microenvironment (17).

Accumulating evidence has suggested that the content of exosomes can be manipulated (18), and these engineered exosomes may serve as ideal vehicles for chemoimmunotherapy involving miRNA/vaccine delivery. For instance, several clinical trials have been performed thus far that have involved immunizing patients with exosomes released in vitro by their own DCs loaded with tumor Ag-derived peptides (19). In another study, the proteomic comparative analysis of DC-derived EVs revealed the presence of the immune regulatory molecule PD-L1 on small EVs that were similar in size to exosomes (20). These findings provide evidence that DC-derived exosomes play a role in the mechanisms associated with immune checkpoint inhibitors in cancer treatment.

Overall, the current study significantly adds to our knowledge of the role of exosomes as important mediators of the innate immune system (21). The evidence that receptors can be transferred from cell to cell through exosomes to induce functional changes in recipient cells can be therapeutically exploited by the application of exosomes as valuable vectors that can be loaded with specific proteins and delivered to target cells. In addition, the corresponding signaling pathways involved in exosome biogenesis, trafficking, and exocytosis may become feasible targets for the clinical treatment of inflammatory diseases and cancer immunotherapy.

DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


