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Exosomes Derived from IL-10-Treated Dendritic Cells Can Suppress Inflammation and Collagen-Induced Arthritis[1]

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We have demonstrated previously that local, adenoviral-mediated gene transfer of viral IL-10 to a single joint of rabbits and mice with experimental arthritis can suppress disease in both the treated and untreated contralateral joints. This contralateral effect is mediated in part by APCs able to traffic from the treated joint to lymph nodes as well as to untreated joints. Moreover, injection of dendritic cells (DC) genetically modified to express IL-4 or Fas ligand was able to reverse established murine arthritis. To examine the ability of exosomes derived from immunosuppressive DCs to reduce inflammation and autoimmunity, murine models of delayed-type hypersensitivity and collagen-induced arthritis were used. In this study, we demonstrate that periarticular administration of exosomes purified from either bone marrow-derived DCs transduced ex vivo with an adenovirus expressing viral IL-10 or bone marrow-derived DCs treated with recombinant murine IL-10 were able to suppress delayed-type hypersensitivity responses within injected and untreated contralateral joints. In addition, the systemic injection of IL-10-treated DC-derived exosomes was able suppress the onset of murine collagen-induced arthritis as well as reduce severity of established arthritis. Taken together, these data suggest that immature DCs are able to secrete exosomes that are involved in the suppression of inflammatory and autoimmune responses. Thus DC-derived exosomes may represent a novel, cell-free therapy for the treatment of autoimmune diseases. ***The Journal of Immunology,*** **2005, 174: 6440–6448.**

The autoimmune disease, rheumatoid arthritis (RA),[4] is a debilitating disease characterized by chronic inflammation of the distal diarthroidial joints (1–3). Once RA is established, the affected joints exhibit inflammatory cell infiltration and synovial hyperplasia that contribute to the progressive degradation of cartilage and bone, resulting in the complete loss of normal joint function. Recently, specific biological agents able to inhibit the proinflammatory cytokines IL-1 and TNF-α have been shown to be therapeutic for treating RA. However, if treatment with the IL-1 and TNF inhibitors is terminated, then disease progression and severity of symptoms return (3). Thus, there is still need for an effective therapy able to reverse the course of arthritis progression following a single or infrequent treatment.

We and others (4–6) have demonstrated that local, adenoviral-mediated gene transfer of viral IL-10 (vIL-10) to a single joint of rabbits and mice with experimental arthritis can suppress disease in both the treated and untreated contralateral joints. This contralateral effect has been observed following both in vivo and ex vivo delivery of a variety of different therapeutic genes in several different animal models of arthritis (6–14). However, the immunological mechanism by which local gene transfer to one joint can distribute antiarthritic effects to untreated joints is currently poorly understood. Recent analysis of the effect suggests that APCs are able to traffic from the treated joint to not only the draining lymph nodes but also untreated joints, suggesting a role for APC in the generation and systemic dissemination of local anti-inflammatory effects (6, 8, 9, 15). We and others (9, 12, 15) have also shown that systemic administration of dendritic cells (DC), genetically modified to express IL-4 or Fas ligand (FasL), was able to reverse established murine collagen-induced arthritis (CIA). These results suggest that immature or certain genetically modified DCs are able to suppress or reverse the course of autoimmune arthritis progression in a murine model.

The release of small lipid vesicles, termed exosomes, by a variety of cells types, including DCs, has been well documented. Exosomes are small membrane-bound vesicles (40–100 nm) that are secreted in the extracellular medium by many cells of hemopoietic origin (16–18) as well certain nonhemopoietic cell types (19). Exosomes are formed by inverse membrane budding into the lumen of an endocytic compartment, which results in the formation of multivesicular intracellular structures or multivesicular bodies. Fusion of the multivesicular bodies with the plasma membrane leads to the release of internal vesicles (exosomes) into the medium. The protein content of exosomes varies depending on the cell type from which they were derived but contains both membrane-associated as well as cytosolic proteins (17, 19–21).

DC-derived exosomes have been characterized extensively at the ultrastructural and protein levels (22–27). However, the in vivo function of DC-derived exosomes remains unclear. Recent experiments suggest that DC-derived exosomes are able to stimulate Ag-specific T cell responses through their ability to interact or fuse

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; vIL-10, viral IL-10; DC, dendritic cell; FasL, Fas ligand; CIA, collagen-induced arthritis; BM-DC, bone marrow-derived DC; DTH, delayed-type hypersensitivity; MHC I, MHC class I; MHC II, MHC class II; eGFP, enhanced GFP; TEM, transmission electron microscopy; KLH, keyhole limpet hemocyanin; exo/vDC-10, exosomes from the Ad-vIL-10 transduced DC.

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with endogenous or follicular DCs. Exosomes may fuse with or be internalized into endogenous DCs and macrophages to transfer class II molecules, as well as possibly other proteins, to the DCs and thus indirectly regulate T cell function. Alternatively, DCs also have been shown to secrete exosomes that carry peptide-loaded MHC molecules that can stimulate T cell proliferation in vitro, suggesting that exosomes alone may be able to present Ag to T cells (28, 29). When used as a tumor vaccine, DC-derived exosomes loaded with acid-eluted tumor peptides eradicated established tumors in mice (30). On the basis of these and other data, two different clinical trials have been initiated using exosomes derived from tumor peptide-pulsed DCs (31, 32).

The ability of DCs to induce or suppress immune responses is dependent on several factors including Ag exposure, state of maturity, and culture conditions (33, 34). Interestingly, it appears that exosomes may have a similar dual capability as well. Several reports have suggested that exosomes can suppress as well as stimulate immune responses (35–38). For example, small particles produced by rat intestinal epithelial cells cultured in the presence of IFN-γ and digested OVA were able to induce the Ag-specific tolerance of that same Ag after injection into mice (38). It has also been shown that both T cells and melanoma tumor cells are able to generate exosomes expressing FasL, which can induce the apoptosis of T cells that would otherwise counteract tumor growth (36, 37).

In this study, we have assessed the in vitro and in vivo immunosuppressive capacity of bone marrow-derived DC (BM-DC)-derived exosomes. Periarticular administration of exosomes derived from BM-DCs that were either transduced ex vivo with an adenovirus expressing vIL-10 or treated with recombinant murine IL-10 was able to suppress murine delayed-type hypersensitivity (DTH) responses within injected and untreated contralateral footpads. In addition, the systemic injection of IL-10-treated exosomes was able to suppress the onset of murine CIA. Collectively, these data suggest that immature DCs are able to secrete exosomes that are involved in the suppression of inflammatory responses. Given that exosomes are unable to change their phenotypes following purification and are stable, DC-derived exosomes represent a novel therapeutic approach for the treatment of inflammatory and autoimmune diseases.

Materials and Methods

Mice

Female C57BL/6 (H-2K^b) mice and male DBA1/LacJ (H-2K^q) mice, all 7–8 wk of age, were purchased from The Jackson Laboratory. Animals were maintained in a pathogen-free animal facility at the University of Pittsburgh Biotechnology Center (Pittsburgh, PA).

Generation and culture of BM-DCs

BM-DCs were generated as described previously (9). Briefly, bone marrow was harvested from mouse tibias and femurs and passed through a nylon mesh to eliminate small pieces of bone and debris. Contaminating erythrocytes were lysed with 0.83 M NH_4Cl buffer, and lymphocytes were depleted with a mixture of Abs (RA3-3A1/6.1, anti-B220; 2.43, anti-Lyt2; and GK1.5, anti-L3T4; all from American Type Culture Collection) and rabbit complement (Accurate Chemical and Scientific) on day 0. The cells were then cultured for 24 h in complete medium (RPMI 1640 containing 10% FBS, 50 μM 2-ME, 2 mM glutamine, 0.1 mM nonessential amino acids, 100 μg/ml streptomycin, and 100 IU/ml penicillin) to remove the adherent macrophages. The nonadherent cells were then placed in fresh complete medium containing recombinant murine GM-CSF (1000 U/ml) and recombinant murine IL-4 (1000 U/ml) on day 1. Cells were cultured for 4 days and harvested for adenoviral transduction or recombinant cytokine treatment on day 5.

For adenoviral infection, 1×10^6 DC/well were plated on 24-well plates, and 5×10^7 PFU of each recombinant adenovirus was added in a total volume of 1 ml of serum-free medium. After incubation for 24 h at 37°C, the cells were collected and washed five times in PBS, and fresh medium was added. On day 7, infected DCs and exosomes were recovered, washed extensively, and injected into animals.

Vector construction and adenovirus generation

Adenoviruses expressing viral IL-10 (Ad.vIL-10) and enhanced GFP (Ad-.eGFP) were constructed, propagated, and titered according to standard protocols as described previously (8). Briefly, the recombinant adenoviruses were generated by homologous recombination in 293 cells expressing Cre recombinase (CRE8 cells), after cotransfection of DNA, an adenovirus 5-derived, E1- and E3-deleted adenoviral backbone (psi5) and pAdlox, the adenoviral shuttle vector. The inserted cDNA sequences are expressed under the human CMV promoter. The recombinant adenoviruses were purified by CsCl gradient ultracentrifugation, dialyzed in sterile virus storage buffer, aliquoted, and stored at −80°C until use. The CRE8 cells were grown and maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS.

Exosome purification

Exosomes were prepared from the cell culture supernatant of day 7 BM-DC cultures by differential centrifugation as described previously (22). Briefly, recovered culture supernatant from each BM-DC culture was subjected to three successive centrifugations at $300 \times g$ (5 min), $1,200 \times g$ (20 min), and $10,000 \times g$ (30 min) to eliminate cells and debris, followed by centrifugation for 1 h at $100,000 \times g$. To remove excess serum proteins, the exosome pellet was washed with a large volume of PBS, centrifuged at $100,000 \times g$ for 1 h, and finally resuspended in 120 μl of PBS for further studies. The exosomes were quantified by a micro Bradford protein assay (Bio-Rad). Each batch was standardized by protein content, and 1 μg was suspended in 20 μl of PBS for in vivo mouse studies. For MHC class II (MHC II) adsorption, 100 μl of washed anti-mouse MHC II paramagnetic beads (Miltenyi Biotech) were incubated with prediluted exosomes (1 μg/20 μl) for 1 h, 4°C with gentle shaking. After magnetic separation, the fraction not retained in the microcentrifuge tube was adjusted to the original volume with PBS. The freeze/thaw of prediluted exosomes was performed by three separate rounds of snap freezing in a dry ice/ethanol bath and subsequent warming in 37°C bath. Contaminating concentrations of IL-10 and vIL-10 in final exosome preparations were determined by IL-10 ELISA (Endogen).

Delayed-type hypersensitivity

On day 0, mice were sensitized by s.c. injection of 100 of μg Ag (OVA) emulsified 1:1 in CFA (Difco). Two weeks later, presensitized mice were given injections in one rear footpad with either 1×10^6 treated DCs (in 50 μl of PBS) or 1 μg of purified exosomes derived from each experimental DC group (in 50 μl of PBS). The contralateral footpads were injected with equal volumes of saline. One day later, the mice were challenged in both rear footpads by injecting 20 μg of Ag dissolved in 50 μl of PBS, and the footpads were measured with a spring-loaded caliper (Dyer) 24, 48, and 72 h later. The results were expressed as the difference in size due to swelling (millimeters $\times 10^{-2}$).

Collagen-induced arthritis

By stirring overnight at 4°C, bovine type II collagen (Chondrex) was dissolved in 0.05 M acetic acid at a concentration of 2 mg/ml and emulsified in an equal volume of CFA. The mice were immunized intradermally at the base of the tail with 100 μg of collagen. On day 21 after priming, the mice received an intradermal booster injection of type II collagen in IFA. For the prevention of disease onset study, the mice received injections with exosomes from DCs at day 28 at the time of disease onset. Mice were monitored every other day by an established macroscopic scoring system ranging from 0 to 4 as follows: 0 = normal; 1 = detectable arthritis with erythema; 2 = significant swelling and redness; 3 = severe swelling and redness from joint to digit; and 4 = maximal swelling and deformity with ankylosis. The average of macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 16 per mouse. For the treatment of established arthritis, mice were given injections with 20 μg of LPS i.p. at day 28 to induce synchronous disease onset. Four days after LPS injection (day 32), exosomes from Ad.vIL-10-transduced or rIL-10-treated DCs were injected into the mice with evidence of disease. The in vivo experiments were performed with 10 mice/group and repeated twice to ensure reproducibility.

Electron microscopy

Exosomes were purified by differential centrifugation, 10 μl loaded on a Formvar/carbon coated grid, negatively stained with 10 μl of neutral 1%

aqueous phosphotungstic acid, and viewed using JEOL-1210 computer-controlled, high-contrast, 120-kV transmission electron microscope.

Protein analysis

The cytosol of cells was separated from the total membranes by homogenization in 10 mM triethanolamine, 1 mM EDTA, 10 mM acetic acid, 250 mM sucrose (pH 7.4), and supplemented with CLAP (chymotrypsin, leupeptin, aprotinin, and pepstatin, 100 μM each), by 60 passages through a 25-gauge needle. The supernatant was cleared from nuclei and cell debris by centrifugation at 1,200 $\times$ *g*. Total membranes were recovered in the pellet after centrifugation for 1 h at 100,000 $\times$ *g*. Proteins (10 μg) were then separated on 5–20% gradient SDS-PAGE, transferred onto nitrocellulose, and detected by Western blot using an ECL detection kit (Amersham Biosciences).

FACS analysis

DCs were defined by phenotypic analysis for expression of CD11b, CD11c, CD80, CD86, and MHC class I (MHC I) and MHC II in the majority of the cultured cells (60–95%) by FACScan (BD Biosciences). For exosomes, 30 μg of pelleted exosomes were incubated with 10 μl of 4-μm diameter aldehyde/sulfate latex beads (Interfacial Dynamics) for 15 min at room temperature in a 30 to 100-μl final volume, followed by 2 h with gentle agitation in 1 ml of PBS. The reaction was stopped by 30-min incubation in 100 mM glycine. Exosome-coated beads were washed three times in FACS wash buffer (3% FCS, and 0.1% NaN_3 in PBS) and resuspended in 500 μl of FACS wash. Beads were incubated for 1 h with each primary Ab, followed when necessary by incubation in FITC-conjugated secondary Ab, washed, and analyzed on a FACSCalibur (BD Biosciences). Data acquisition and analysis were performed using Lysis II FACScan software (BD Biosciences).

Mixed lymphocyte reaction

T cells were purified from the spleens of BALB/c mice for in vitro microculture in round-bottom, 96-well plates. In each well, 5×10^4 splenic T cells were seeded with either control C57BL/6-derived DCs or genetically modified C57BL/6-derived DCs (vIL-10, rIL-10, or luciferase). On day 5 of culture, 1 μCi of [^{3}H]thymidine was added to each well 16 h before harvest. Radioactive labeling of proliferating T cells was measured on a microplate beta counter (Wallac).

Statistical analysis

All data were analyzed using the Microsoft Excel software program. Group comparisons were performed using both Student's *t* test and ANOVA.

Results

Characterization of murine BM-DC-derived exosomes

To examine the immunoregulatory role of DC-derived exosomes, DCs were generated from C57BL/6 mouse bone marrow precursors cultured at high density in GM-CSF/IL-4. Exosomes produced by the DCs were then isolated from the culture medium by differential centrifugation and characterized by electron microscopy, Western blot, and flow cytometry (Fig. 1). Ultrastructural analysis of exosome pellets by whole mount transmission electron microscopy (TEM) showed a significant enrichment of the characteristic saucer-shaped exosomes, 40–100 nm in diameter (Fig. 1*A*). Western analysis demonstrated that the DC-derived exosomes were positive for the exosome-associated proteins CD71 and Hsp70 (Fig. 1*B*) but negative for proteins not found in exosomes, such as Hsp90, the invariant chain, and calnexin (data not shown). To further demonstrate the intact vesicular nature of the exosome fraction, exosomes were purified from BM-DCs of eGFP/C57 mice, because the majority of cells within the animal constitutively express the marker protein eGFP. Western analysis of the highly enriched exosome fraction showed a significant level of full-length eGFP, suggesting that the soluble eGFP is encapsulated within the protective environment of the lumen of BM-DC-derived exosomes.

We further examined the surface proteins on the DC-derived exosome fraction by flow cytometry. Exosomes were recovered after the 100,000 $\times$ *g* spin, bound to latex beads, and stained with several mAbs against murine DC-associated leukocytic marker proteins. The surface of exosomes stained positive for high levels of MHC II, with more moderate levels of MHC I, CD11C, CD80 (B7.1), and CD86 (B7.2) detected (Fig. 1*C*). Taken together, these data demonstrate the ability to enrich for intact exosomes that contain many of the markers of DC-derived exosome-associated proteins as described previously (21, 22, 24).

In vitro function of BM-DC-derived exosomes

To test the ability of BM-DC-derived exosomes to suppress T cell proliferation, the effect of adding DC-derived exosomes to a MLR

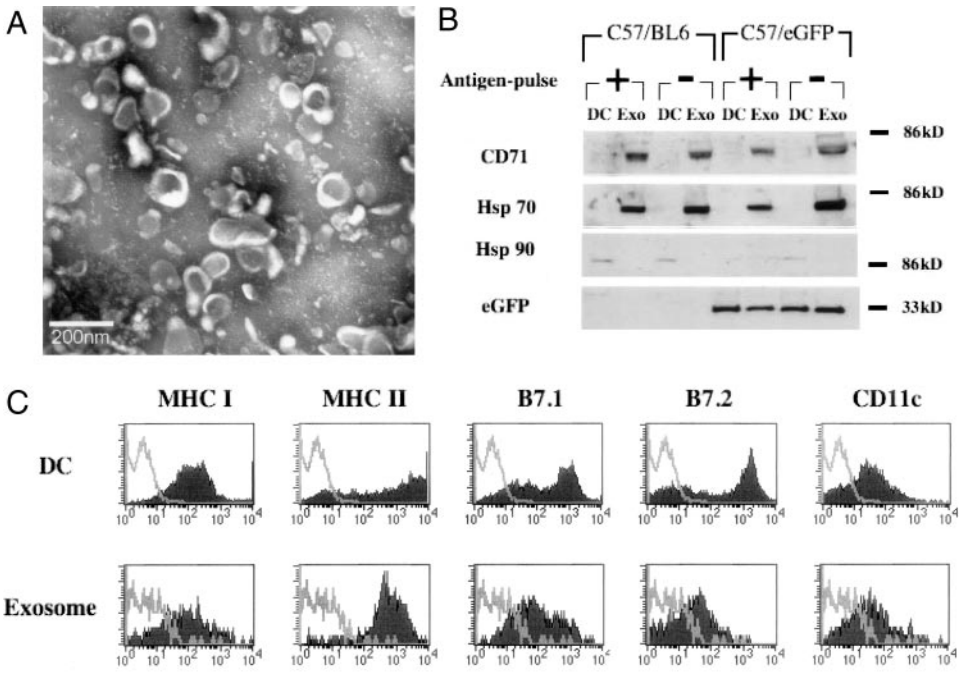

FIGURE 1. Characterization of exosomes from BM-DCs. *A*, Whole-mount TEM of exosomes from murine BM-DCs. BM-DCs were grown from lymphocyte-depleted monocytic precursors and cultured in GM-CSF and IL-4 for 7 days. The cell culture supernatants were then sequentially centrifuged to obtain the pellet containing exosomes. The 100,000 $\times$ *g* pellet was washed and analyzed by TEM. Bar, 200 nm. *B*, Western blot analysis of exosome-associated proteins. Ten micrograms of exosomes and BM-DC lysates were separated by SDS-PAGE and analyzed in parallel by Western blotting for the presence of several exosome-associated proteins. *C*, Flow cytometric analysis of murine DC-derived exosomes. BM-DC-derived exosomes were isolated by differential centrifugation, coated onto beads, and stained with FITC-coupled Abs specific for MHC I and II, CD11c, CD80 (B7.1), CD86 (B7.2), or the correspondingisotype controls.

was examined. As a source of potentially immunosuppressive DC-derived exosomes, BM-DCs transduced with an adenovirus expressing the EBV-encoded *IL-10* gene, termed vIL-10, were used. Intra-articular gene transfer of vIL-10 has been shown to suppress inflammation in both rabbit Ag-induced arthritis and murine CIA models (5, 6). As a control, BM-DCs transduced with adenoviral vector expressing luciferase (Ad.Luc) were used. When added to the MLR, the DCs transduced with Ad.vIL-10 were able to almost completely suppress T cell proliferation, as measured by [^{3}H]thymidine incorporation, whereas addition of nontransduced DCs displayed little or no effect (Fig. 2*A*). Exosomes secreted by BM-DCs infected with Ad.vIL-10 exhibited a moderate 4-fold decrease in T cell proliferation (Fig. 2*B*). These data suggest that exosomes isolated from the immunosuppressive vIL-10-expressing DCs are able to block T cell proliferation and that exosomes derived from unmodified BM-DCs may themselves harbor partial anti-inflammatory properties.

Exosomes can suppress inflammation in a DTH model

To investigate the anti-inflammatory effect of BM-DC-derived exosomes in vivo, a DTH model in C57BL/6 mice was used. Previously, we have used this model to show that injection of Ad-.vIL-10 into one hind footpad results in suppression of inflammation in both the injected and contralateral footpad. Moreover, we have shown by adoptive transfer experiments that the contralateral effect observed following local Ad.vIL-10 delivery in the DTH model was conferred by endogenous APC. Groups of sensitized mice were given injections in the right rear footpad with either untreated or genetically modified DCs or the exosomes derived from the culture media of these cells. The contralateral footpads received a saline injection of a similar volume. After 12 h, each footpad was challenged with 20 μg of keyhole limpet hemocyanin (KLH), and footpad swelling was monitored at 24, 48, and 72 h after disease induction. As shown in Fig. 3, the DTH response in saline control animals was acute, with the average increase in paw thickness >2 mm. However, footpad swelling was reduced by >50% in the injected footpads of mice receiving 1×10^6 BM-DCs transduced with Ad.vIL-10. A reduction of inflammation (40%) was also observed in the saline-treated contralateral footpads of these same animals. Interestingly, injection of 1 μg of secreted

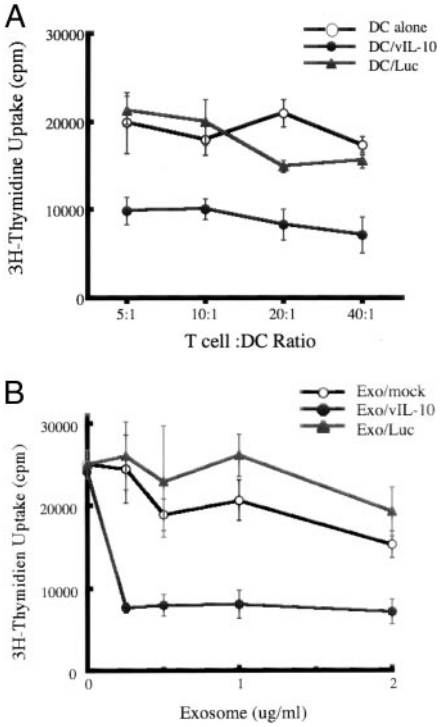

FIGURE 2. Analysis of function of BM-DC-derived exosomes in an MLR. *A*, The 5×10^5 cells/ml T cells from BALB/c (H-2kd) spleen were stimulated with C57BL/6 (H-2kb) bone marrow DCs noninfected or infected either Ad-vIL-10 or Ad-Luc at serial ratio. After 4 days of culture, 1 μCi of [^{3}H]thymidine was added in each well 16 h before harvest. *B*, Exosomes were isolated from C57BL/6 DCs that were noninfected or infected with either Ad-vIL-10 or Ad-Luc. The serial concentrations of exosomes were added into culture of T cells isolated from BALB/c spleen and naive DCs from C57BL/6 at 10:1 ratio. T cell proliferation was determined by [^{3}H]thymidine incorporation after 5 days of culture. Luc, Luciferase.

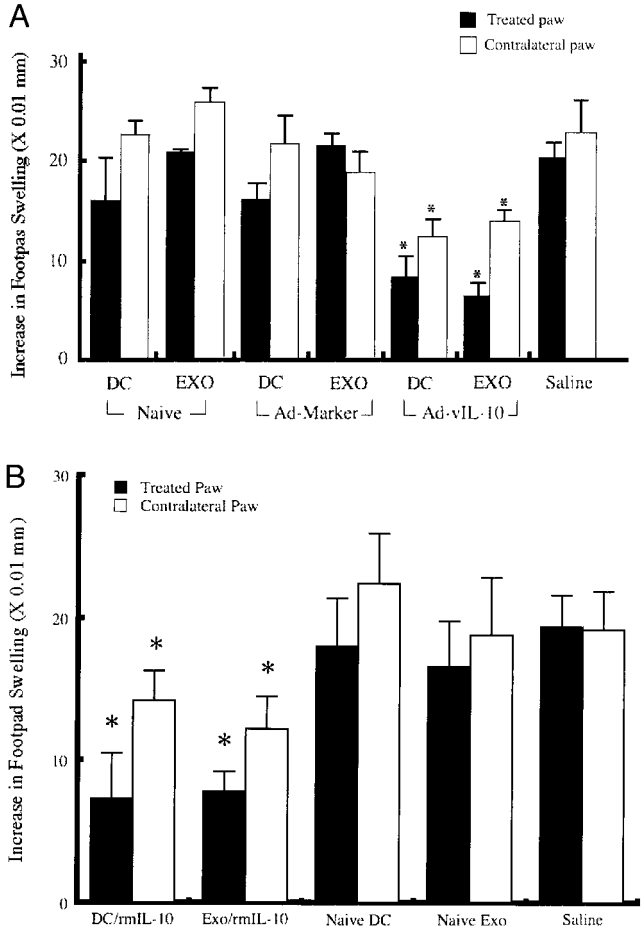

FIGURE 3. Suppression of the DTH response by DCs and DC-derived exosomes. *A*, Suppression of DTH response in Ad-vIL-10-transduced DCs and exosomes derived from the DC/vIL-10. Groups of mice were sensitized to KLH in CFA by s.c. injection on day 0. Two weeks later, sensitized mice received injections into the right footpad with 1×10^6 untreated DCs, DCs transduced with 50 multiplicity of infection of Ad-Luc, DCs transduced Ad.vIL-10, or 1 μg of exosomes derived from these cells. The contralateral joints were injected with a similar volume of saline. The mice were then challenged with 20 μg of Ag in PBS 12 h later, and footpad swelling was measured at 24-, 48-, and 72-h time points. *B*, Suppression of DTH reaction with recombinant murine IL-10-treated BM-DCs and exosomes derived from DCs treated with recombinant murine IL-10. Day 5 BM-DCs were treated with 1 μg/ml recombinant murine IL-10 protein for 24 h. After washing, treated DCs were cultivated for an additional 48 h before harvest. Exosomes were isolated from either naive DCs or recombinant murine IL-10-treated DCs and injected into the right footpad of KLH-immunized mice. After challenge of KLH into both footpads, swelling of footpad was measured at 24-, 48-, and 72-h time points. *, Significance at $p < 0.01$. rmIL-10, Recombinant murine IL-10.

exosomes derived from Ad.vIL-10-tranduced BM-DCs was even more protective, suppressing paw swelling by 65% compared with saline control mice. Furthermore, a significant reduction was also observed in the footpads contralateral to the Ad.vIL-10/exosome-treated joints (Fig. 3*A*). No significant reductions in footpad swelling were observed following administration of Ad.Luc/DCs, Ad-.Luc/exosomes, untreated DCs, or exosomes from untreated DCs. These data suggest that exosomes derived from Ad.vIL-10-transduced BM-DCs can suppress DTH in both treated and untreated contralateral footpads when delivered locally to sensitized mice.

rIL-10-treated-DC-derived exosomes are immunosuppressive

Although the experiments performed above suggest that exosomes derived from Ad.vIL-10-transduced DCs are immunosuppressive, it is possible that a low level of Ad.vIL-10 or vIL-10 protein contaminated the exosome preparation. To show that adenovirus infection or vIL-10 protein contamination did not contribute to the observed effects, BM-DCs were treated with recombinant murine IL-10 protein and the generated exosomes tested in vivo using the DTH model in C57BL/6 mice (Fig. 3*B*). The exosomes derived from recombinant murine IL-10-treated DCs produced a strong immunosuppressive effect 48 h postchallenge, as demonstrated by a 6-fold reduction in paw swelling in the treated paws and a 3-fold reduction in the untreated contralateral paws. Taken together, these results demonstrate that exosomes derived from murine IL-10-treated BM-DCs can suppress DTH in both treated and untreated contralateral footpads, effectively ruling out adenovirus contamination as the mechanism for this effect. It is also important to note that no rIL-10 protein was detected in the exosome preparations by ELISA.

Membrane disruption causes loss of immunosuppressive ability of exosomes

To confirm that the exosomes present in the enriched 100,000 $\times$ *g* pellet fraction were important for conferring the therapeutic effects in the DTH model, the requirement for intact exosomes particles for efficacy was examined. Initially, we demonstrated by electron microscopy that the integrity of exosomes was disrupted by four cycles of freeze/thaw (Fig. 4*A*). In addition, the soluble, exosome-associated protein Hsc70 was not detected in the freeze/thaw-treated exosome fractions, whereas it was associated with untreated exosomes (Fig. 4*B*). To test whether exosomes require an intact membrane to suppress the DTH response, intact or freeze/thawed exosomes from DCs transduced with Ad-vIL-10 were injected into the footpad of one hind paw of KLH-immunized mice (Fig. 4*C*). After 24 h, each footpad was boosted with KLH, and the extent of footpad swelling was measured. Although reduction of footpad swelling was observed in the group treated with intact exosomes from the Ad-vIL-10 transduced DC (exo/vIL-10), the group injected with freeze/thaw-treated exo/vIL-10 showed no reduction in paw swelling, similar to the control groups treated with saline or exosomes from control DCs (also see Fig. 7*B*). These results demonstrate that multiple cycles of freeze/thaw disrupted membrane structure of exosomes, thereby eliminating the immunosuppressive ability of exosomes in the DTH model. Thus, intact vesicles appear to be required for the observed suppressive effect.

MHC II-containing exosomes required for suppression of the DTH response

To determine whether the exosomes able to suppress the DTH response indeed were derived from DCs, the effect of depleting class II-positive particles on suppression of the DTH response was examined. Exosomes from Ad-vIL-10-transduced BM-DCs were divided into four samples for pretreatment before injection into sensitized mice (Fig. 5*A*). The first exosome sample was preadsorbed with paramagnetic beads specific for murine MHC II, whereas the second sample was preadsorbed with paramagnetic beads specific for NK1.1, a cell surface molecule not present on DC-derived exosomes. The third sample was subjected to multiple cycles of freeze/thaw, whereas the fourth sample was left untreated. The exosome samples were then injected into one hind footpad of mice, and 12 h later, DTH-induced footpad swelling in both hind footpads was measured over the next 72 h. Similar to previous experiments, exosomes from control DCs had no effect on footpad swelling, whereas Ad.vIL-10/exosomes were able to dramatically block DTH in both the injected and untreated contralateral footpads. The exosome preparation preadsorbed to the NK1.1 beads exhibited immunosuppressive activity. However, preadsorption of the Ad.vIL-10/exosome sample with class II beads abrogated ~100% of the activity in vivo. Importantly, the

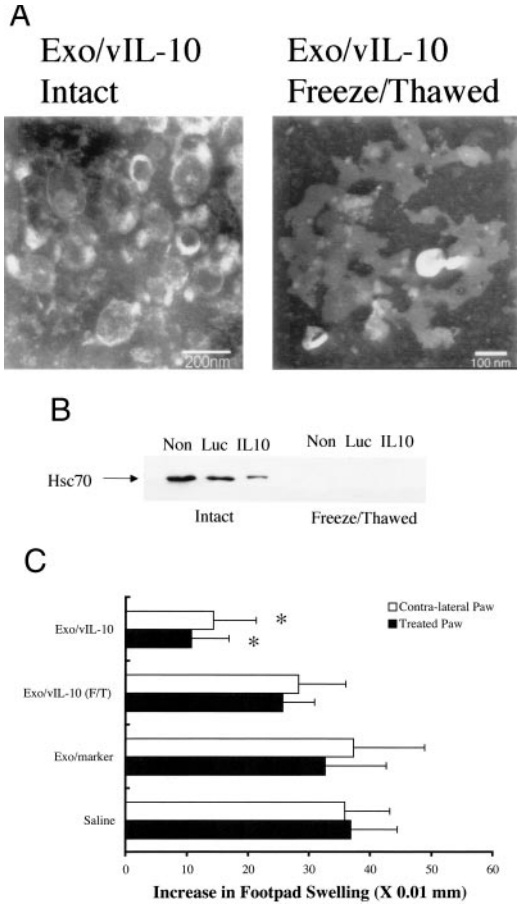

FIGURE 4. Membrane disruption of exosomes abrogates the immunosuppressive effects. *A*, Whole-mount TEM of intact or freeze/thawed exosomes from BM-DCs transduced with Ad-vIL-10. *B*, Western Blot against anti-Hsc70 for intact or freeze/thawed exosomes from BM-DCs transduced with Ad-vIL-10. Exosomes from either nontransduced DCs or DCs transduced with Ad-Luc or Ad-vIL-10 were isolated and subjected to multiple cycles of freeze/thaw. Total of 5 μg of intact or freeze/thawed exosomes were separated by SDS-PAGE and analyzed in parallel by Western blotting for Hsc70. *C*, Analysis of membrane-disrupted exosomes in the DTH model. Exosomes were isolated from either Ad-vIL-10- or Ad-Luc-transduced mouse bone marrow DCs. After several cycles of freeze/thaw, 1 μg of intact or disrupted exosomes was injected into one hind paw of the KLH-immunized mouse. After boost injection of KLH, swelling of both hind paws was measured at 48-h time point. $*$, Significance at $p < 0.01$. Luc, Luciferase; F/T, freeze/thaw.

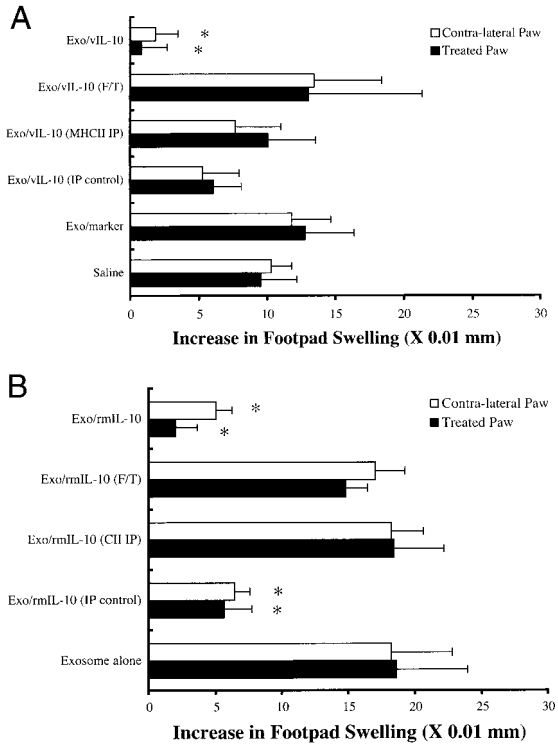

FIGURE 5. MHC II depletion abrogates the immunosuppressive effect of exosomes in the DTH model. Before footpad injection, a single, large-scale preparation of exosomes derived from BM-DCs that were transduced with Ad.vIL-10 (*A*) or treated with recombinant murine IL-10 (*B*) were diluted into parallel experimental groups. One group was preincubated with either anti-MHC II or anti-NK1.1 paramagnetic beads, and another group was disrupted by a series of freeze/thaw cycles. The effect of the exosome fractions on footpad swelling is shown. *, Significance at $p < 0.01$. F/T, Freeze/thaw;IP, immunoprecipitation; rmIL-10, recombinant murine IL-10.

injection of the actual paramagnetic beads with bound, class II-positive exosomes resulted in immunosuppressive activity similar to that seen with the nonadsorbed Ad-vIL-10 exosomes. Exosomes from recombinant mouse IL-10 protein-treated DCs showed similar results in the DTH response after MHC II depletion (Fig. 5*B*). The results were consistent with each other. The formulation that underwent several freeze/thaw cycles in both the exosomes from Ad-vIL-10- or rIL-10-treated DCs also lost activity. These data suggest that the in vivo anti-inflammatory effects are conferred by MHC II-positive vesicles. Furthermore, it appears that the integrity of the vesicle is also required. Given that the in vivo anti-inflammatory effects also appear to be specific for structurally intact MHC II-positive exosomes, this rules out adenovirus contamination as the mechanism for the effect.

Exosomes can suppress CIA in mice

RA is a debilitating autoimmune disease characterized by chronic inflammation of the distal diarthroidial joints and progressive destruction of cartilage tissue. Similar pathologies as well as inflammation in joints can be induced in the DBA1/lacJ (H-2k^q) strain with injection of bovine type II collagen. To examine the ability of DCs and DC-derived exosomes to treat CIA, DCs were infected with Ad-vIL-10, and the resultant exosomes were injected i.v. into DBA1 mice immunized with bovine type II collagen. Injection was done at day 28, just before disease onset. A single injection of either DC/vIL-10 or exosomes from DC/vIL-10 was able to delay the onset and reduce the severity of arthritis, whereas disease progressed normally in the saline-injected control group (Fig. 6). This

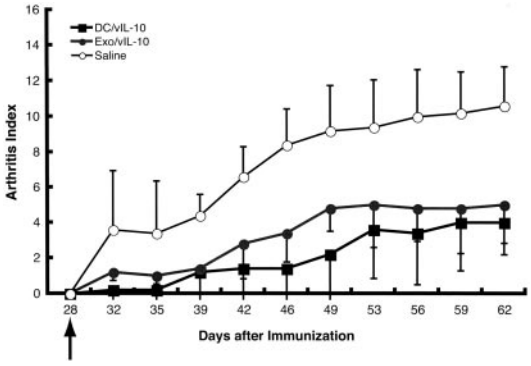

FIGURE 6. Analysis of exosomes derived from DC/vIL-10 in prevention of onset of murine CIA model. Exosomes were isolated from DBA1 mouse bone marrow that was infected with Ad-vIL-10. The purified exosomes were injected i.v. at day 28 (arrow indicates) into the DBA1 mice, which were immunized with bovine type II collagen. Mice were monitored periodically by an established macroscopic scoring system. The macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 16.

result suggests that a single injection of exosomes derived from DCs expressing IL-10 is comparable to an injection of genetically modified parental DCs in preventing onset of CIA.

In addition to the analysis in DC-derived exosomes in the prevention of disease study, exosomes from DC/IL-10 were tested in the mice with established CIA. Exosomes from Ad.vIL-10-transduced or rIL-10-treated DCs were injected i.v. into the mice with established disease (Fig. 7). Although disease suppression in the exo/IL-10-treated group was less than that shown in the prevention study, exosomes from both the Ad.vIL-10-transduced and rIL-10-treated DCs were able to reduce the severity of established disease (Fig. 7*A*). Moreover, freeze/thaw treatment of the exosomes abrogated the therapeutic effect (Fig. 7*B*), whereas direct injection of rIL-10 protein had no effect on disease progression (*C*).

Discussion

Recently, exosomes have been shown to be involved in regulating certain biological processes. In particular, exosomes can be used to transfer proteins to cells through membrane fusion, potentially mediated by tetraspanins and MFG-E8 (39, 40). For example, microvesicles, possibly exosomes, can transfer the HIV coreceptor, CCR5, from CHO cells to CD4$^+$ T cells that do not carry CCR5, thus rendering the T cells susceptible to HIV infection. In addition, exosomes are able to transfer MHC II molecules to follicular DCs, which are normally void of class II proteins (41). Exosomes derived from B cells have been shown to stimulate CD4$^+$ cells in an Ag-specific manner, although the extent of stimulation is lower than that achieved using B cells directly (42). It also has been shown that exosomes produced by DCs pulsed with tumor Ag peptides were able to stimulate an antitumor response in mice as efficiently as the DCs themselves (43). However, the ability of the exosomes carrying a specific class I peptide to stimulate T cell proliferation in culture required unpulsed DCs, suggesting that exosomes transfer the MHC I complex to the DCs (29). It also has been shown that exosomes derived from tumor cells are able to transfer tumor Ags to DCs, presumably through fusion (44). Recently, two different clinical trials have been initiated using exosomes derived from tumor Ag peptide-pulsed DCs (31, 32).

Given the ability of DC-derived exosomes to stimulate immune responses in culture and in vivo, we were interested in determining whether exosomes derived from immunosuppressive DCs were

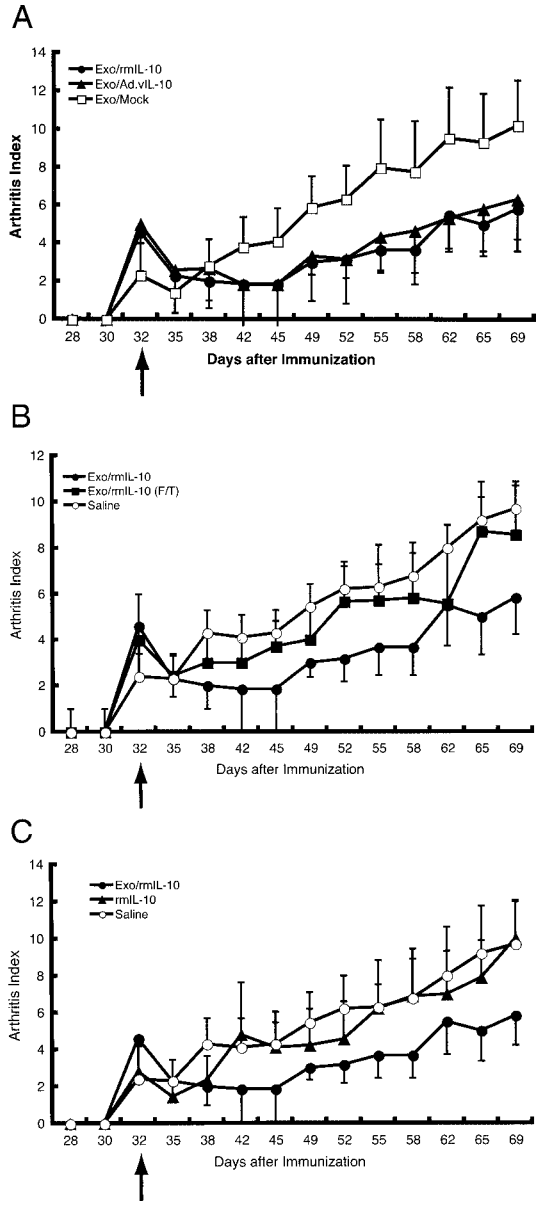

FIGURE 7. Analysis of the therapeutic effect of exosomes derived from DC/IL-10 in an established CIA model. Exosomes were isolated from DBA1 mouse bone marrow DCs that were infected with either Ad.vIL-10 or pulsed with recombinant mouse IL-10 (*A*). Exosomes from rIL-10-pulsed DCs were divided into two groups, and one of them was subjected for three cycles of freeze and thaw to disrupt the membrane (*B*). Exosomes from DC treated with recombinant murine IL-10 were tested in the established CIA mouse compared with direct injection of recombinant murine IL-10 (*C*). The purified exosomes were injected i.v. at day 32 (as indicated by the arrow) into the DBA1 mice, which were immunized with bovine type II collagen and given LPS at day 28. Mice were monitored periodically by an established macroscopic scoring system expressed as a cumulative value for all paws, with a maximum possible score of 16. rmIL-10, Recombinant murine IL-10; F/T, freeze/thaw.

able to suppress inflammation. In this report, we have demonstrated the ability of exosomes derived from DCs either expressing vIL-10 or treated with recombinant murine IL-10 to suppress paw inflammation in a murine DTH model. Interestingly, the injection of DC-derived exosomes as well as DCs was able to suppress inflammation in both the injected footpad and the untreated contralateral footpad. These results are similar to our previous experiments in which injection of an adenovirus expressing vIL-10 into one hind footpad resulted in the treatment of both the injected and the untreated contralateral footpad (5). This observation suggests that both DCs and DC-derived exosomes are involved in conferring the observed contralateral effect. Consistent with this observation is the fact that we have detected luciferase-positive microvesicles in the synovial fluid in both injected and contralateral rabbit knee joints following intra-articular injection of Ad.Luc.

It is important to note that we have demonstrated that intact exosomes are required for conferring the therapeutic effects in the DTH model. Treating exosomes derived from DCs infected with Ad.vIL-10 or from DCs treated with recombinant murine IL-10 with three cycles of freeze/thaw resulted in the complete loss of the therapeutic effect in not only the DTH model but also in established CIA. This result suggests that the observed therapeutic effect in the DTH and CIA models requires intact particles rather than only membranes. Similarly, the observed suppression of inflammation or autoimmunity by the exosome preparation could be abrogated by sonication (S. H. Kim, N. Bianco, and P. D. Robbins, unpublished material), consistent with intact vesicles being important for mediating the biological effects. The results with treatment of DCs with rIL-10 also demonstrate that it is not necessary to infect DCs with adenoviral vectors to generate immunosuppressive exosomes. Thus, the observed therapeutic effects are not due to contaminating adenovirus in the exosome preparation, a possibility that was unlikely based on the fact that at least 10^5 PFU of Ad-.vIL-10 are required for a therapeutic effect in the DTH model. The ability to generate immunosuppressive exosomes from DCs without the need for gene transfer should facilitate their clinical application.

In addition to suppressing DTH, our exosome preparations were able to suppress the onset and severity of CIA as well as partially reverse established disease. We demonstrated that a single injection of exo/Ad.vIL-10 or exo/rIL-10 conferred these effects over an extended period. Of all the different gene- and cell-based therapies currently being tested in murine CIA, systemic injection of exosomes appears to be the most effective. In fact, recent results suggest that exosomes derived from DCs genetically modified to express a membrane-bound IL-4 completely reverse established disease with disease-free status maintained for an extended period (S. H. Kim, N. Bianco, A. Morelli, and P. D. Robbins, unpublished material). However, it is still unclear whether treatment with specific DC-derived exosomes results in tolerance induction to specific Ags. Interestingly, control exosomes derived from DC control cells also were able to confer a weak therapeutic effect, suggesting that optimizing the growth conditions of DCs could result in the generation of immunosuppressive exosomes to treat arthritis. Unlike DCs, which can undergo phenotypic changes following injection, exosomes appear to be static, presumably reflecting the phenotype of the DCs at the time of release. Thus, the use of exosomes derived from DCs made to be immunosuppressive may be safer and more effective than the use of modified DCs.

In preliminary experiments, the ability of both DCs and exosomes to suppress the DTH response appears to be MHC II dependent, but MHC I independent as demonstrated using DCs, genetically modified to express FasL, and exosomes from class I- and II-deficient mice (S. H. Kim, N. Bianco, A. Morelli, and P. D. Robbins, unpublished material). These data were confirmed by the observation that syngeneic, but not allogeneic, DC-derived exosomes were able to suppress the DTH response. We also have shown that exosomes from FasL-expressing DCs pulsed with specific Ag, but not unrelated Ag, were able to suppress the inflammatory responses, suggesting that exosomes confer Ag-specific suppression (S. H. Kim, N. Bianco, A. Morelli, and P. D. Robbins, unpublished material).

The mechanism(s) through which exosomes function to suppress DTH as well as CIA is unclear. Presumably the DCs expressing vIL-10 are able to regulate T cell responses directly by the down-regulation of costimulatory molecules such as B7.1 and B7.2. However, exosomes are less effective in the regulation of T cells responses in vitro compared with DCs, suggesting that a different, indirect mechanism may be at work in vivo. It is possible that exosomes are able to bind and perhaps fuse with endogenous cells, macrophages, or APC, to modulate their activity. However, a recent report suggests that DC-derived exosomes are internalized into macrophages and DCs (45). It is also possible that the exosomes function at several levels such as fusion and direct interaction with T cells. Interestingly, exosomes from DCs expressing vIL-10 showed decreased levels of heat shock protein Hsc70 (see Fig. 4*B*). Hsp70 is known to convert T cell tolerance to autoimmunity in vivo (46), and Hsc70 showed accumulative expression in RA synovial tissue (47).

Recently, we have initiated adoptive transfer experiments to examine the mechanism of action of the DC-derived exosomes. In these experiments, different cell populations from spleens and lymph nodes of mice treated with DCs or DC-derived exosomes were transferred to mice before injection of the Ag used to induce a DTH response. In preliminary experiments, both T cells and DCs from exosome-treated mice were able to suppress significantly the DTH response following injection into the footpad. This suggests that DC-derived exosomes are able to modulate, either directly or indirectly, the activity of both endogenous DCs and T cells, rendering them able to confer anti-inflammatory effects. Taken together, these results imply that DC-derived exosomes suppress inflammation and autoimmunity through a class II-dependent pathway in an Ag-specific manner by modulating the activity of both endogenous T cells and APCs. The strong immunosuppressive effects conferred by exosomes suggest that they could have a significant therapeutic effect in clinical studies.

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Disclosures

P. D. Robbins is a member of the Scientific Advisory Boards for Tissuegene, Inc., and Orthogen AG, which are developing biological therapies for arthritis.

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